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ON THE NATURE OF THE SUPERFICIAL LAYER IN CELLS AND ITS RELATION TO THEIR PERMEABILITY AND TO THE STAINING OF TISSUES BY DYES.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

(Received for publication, October 19, 1907.)

I. INTRODUCTION.

It is a truism that ere a tissue or cell can stain in a dye the dye and the elements which stain must first be brought into physical contact. The importance of the *solubility* of the dye in various tissue elements is evident in this connection and has been especially insisted upon by Witt,¹ Georgievics² and A. Fischer.³ Nevertheless as regards the normal protein constituents of cells and tissues the solubility of the dye in water may probably be considered as a measure of its solubility in the protoplasm of the cells, for even if we consider the protoplasm as a polyphasic system consisting essentially of colloid-poor, water-rich phases in equilibrium with colloid-rich, water-poor phases,⁴ yet the common constituent of these phases is water, by means of solution in which the distribution of the dye may be accomplished. The case is, however, far different for the fatty constituents of cells and for protein and other constituents which may be enveloped in a fatty layer, for in order to stain these the

¹ O. Witt: Theorie d. Färbeprocesses, *Färbezeitung* (1890-1), quoted after Gustav Mann, *Physiological Histology*, Oxford, p. 320, 1902.

² G. v. Georgievics: *Sitzungsber. d. Akad. d. Wiss.*, Wien, ciii, p. 589, 1894; G. v. Georgievics and E. Löwy: *Ibid.*, civ, p. 309, 1895, quoted after Gustav Mann, *loc. cit.* p. 331.

³ A. Fischer: *Fixirung, Färbung und Bau des Protoplasmas*, 1899.

⁴ W. B. Hardy: *Proc. Roy. Soc.*, lxvi, p. 95, 1900; *Journ. of Phys. Chem.*, iv, pp. 235 and 254, 1900; *Journ. of Physiol.*, xxxiii, p. 251, 1905; Bütschli: *Untersuchungen über microscopische Schäume und das Protoplasma*, Leipzig, 1892; *Arch. f. Entwicklungsmechanik*, xi, p. 499, 1901.

dye must be soluble in lipoids. The importance of this fact has been realized by Overton¹ and he has used it in support of his hypothesis that the superficial layer of cells is lipid in nature.² Overton investigated the distribution-coefficients of dyes between water and lecithin, protagon and cerebrin, using these lipoids either in the form of suspensions or in solution in benzene, and also between water and olive oil, tripalmitin, tristearin, triolein and other lipoids. He used only the salts of the dyes in neutral solution and found that the salts of the basic anilin dyes were very soluble in the lipoids investigated, while the sulphonated color-acids and sodium carminate were but sparingly soluble in lipid or not at all. He believes that animal and plant cells are similarly readily stained by basic anilin dyes and only slowly or not at all by the sulphonic-acid group. Were this so it would indeed be a curious fact that red blood corpuscles, which contain a notably high percentage of lipid, select orange G, which is a sulphonic acid, from Ehrlich's triacid mixture rather than methyl green, which is a basic dye. It is a well-known fact that the coefficient of distribution of a base or of an acid between two solvents greatly depends upon the conditions under which it is measured: thus, to quote a classic example, succinic acid in the free state is six times more soluble in ether than in water, that is, on shaking up a solution of succinic acid with water and ether it will distribute itself between the two solvents in such a way that the concentration of the succinic acid in the ethereal layer is always six times the concentration of the succinic acid in the watery layer (provided the temperature is constant).³ If, however, alkali, for example sodium hydroxide, be added to the mixture sodium succinate will be formed and this is more soluble in the water than in the ether, so that now a greater proportion of the succinic acid will be present in the watery layer than in the ethereal layer.

In the case of dyes we should expect to find similar phenomena,

¹ E. Overton: *Jahrb. f. wiss. Botanik*, xxxiv, p. 669, 1900.

² E. Overton: Ueber die allgemeinen osmotischen Eigenschaften der Zelle, ihre vermuthlichen Ursachen und ihre Bedeutung für die Physiologie, *Vierteljahrsschrift der naturforsch. Gesellsch. in Zürich*, xlv, p. 88, 1899.

³ Berthelot et Jungfleisch: *Ann. de chim. et de phys.* (4), xxvi, p. 396, 1872; Berthelot: *ibid.*, p. 417.

more especially as the dyes commonly used for staining tissues are usually pseudo-acids or pseudo-bases and undergo an intra-molecular change upon the addition of acid or alkali.¹ Provided that the presence of acid or alkali influences the lipid-solubility of dyes this fact may be expected to play a considerable part in determining the staining-reactions of the lipid elements in tissues. The dyes commonly used in histology are frequently used in acid or alkaline solution and, moreover, the proteins in solution in the protoplasm of cells can act either as weak acids or as weak bases² and by so doing may be expected to influence the staining of adjacent elements of a lipid character. From the staining-reactions of elements which are known to consist of lipid material we might therefore deduce the nature, whether predominantly acid or predominantly basic, whether combined or uncombined, of the proteins in solution in the surrounding intra- or extracellular fluids. It therefore appeared to me to be of considerable interest and of importance in the interpretation of the staining-reactions of tissues to determine whether, and in what sense, acids or alkalis influenced the relative solubilities of dyes in lipoids and in water.

II. EXPERIMENTAL.

The following is a table of the dyes employed. Many of these dyes are amphoteric, that is, can act either as acids or as bases; hence when, for example, it is stated in the accompanying table that a dye is basic it is not meant to imply that it acts exclusively as a base but that in case both functions are present the basic function predominates over the acid function:

¹ Hantsch: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 575, etc., 1899.

² Strecker: *Liebig's Ann. d. Chem.*, cxlviii, p. 87, 1886; O. Loew: *The Energy of Living Protoplasm*, London, p. 21, 1896; Bredig: *Zeitschr. f. Elektrochemie*, vi, p. 33, 1899; Winkelblech: *Ueber amphotere Elektrolyte und innere Salze*; Leipziger Dissertat., 1901, quoted after Mann, *Chemistry of the Proteids*, p. 208, 1906; *Zeitschr. f. physikal. Chem.* xxxvi, p. 546, 1904; J. Loeb: *Univ. of Calif. Publ., Physiol.*, i, p. 149, 1904; Walker: *Zeitschr., f. physikal. Chem.*, xlix, p. 82, 1904; W. B. Hardy: *Journ. of Physiol.*, xxxiii, p. 251, 1905; Gustav Mann: *Chemistry of the Proteids*, pp. 145 and 208, 1906; T. Brailsford Robertson: *Journ. of Physical Chem.*, x p. 524, 1906, and xi, p. 437, 1907; this *Journal*, ii, p. 317, 1907.

TABLE 1.

Name of dye.	Structure.	Character.
Iodine eosin (Gruebler).....	phenyl-methane group	acid
Erythrosin (pur. Gruebler).....	phenyl-methane group	acid
Acid fuchsin (Saurefuchsin n. Weigert. Gruebler)	phenyl-methane group	acid
Rosolic acid.....	phenyl-methane group	acid
Gentian violet (Gruebler).....	phenyl-methane group	basic
Crystal violet (Klönne and Muller).....	phenyl-methane group	basic
Methyl violet B (Gruebler).....	phenyl-methane group	basic
Methyl green (Gruebler).....	phenyl-methane group	basic
Dahlia (Gruebler).....	phenyl-methane group	basic
Rhodamin (B extra).....	phenyl-methane group	basic
Pyronin (Gruebler).....	phenyl-methane group	basic
Thionin (pur. Gruebler).....	quinone-imide group	basic
Safranin (Spiriti. Gruebler).....	quinone-imide group	basic
Neutral red (zu Injekt. i. vital. Gew. n. Ehrlich. Gruebler).....	quinone-imide group	basic
Methylene blue (rectif. n. Ehrlich. Gruebler)...	quinone-imide group	basic
Tropaeolin 000.....	azo group	acid
Orange G (Gruebler)	azo group	acid
Methyl orange (Kahlbaum).....	azo group	acid
Bordeaux red (Gruebler).....	azo group	acid
Congo red (Gruebler).....	azo-group	acid
Bismarck brown (Gruebler).....	azo group	basic
Sudan III (Gruebler).....	azo group	neutral
Scharlach R (Michaelis, Gruebler).....	azo group	neutral
Wool black (Gruebler).....	azo group	
Azure II (Gruebler).....		basic
Azure II eosin (Gruebler)		dye-salt
Picric acid (Kahlbaum).....	nitro group	acid
Lacmoid.....	derivative of resorcin	
Sulphindigotate of sodium (Kahlbaum).....		acid
Haematin (Merck).....	contains quinonoid group	
Orcein (Gruebler).....	not determined	acid
Carminic acid (Pur. Gruebler).....	not determined	acid
Carmin (Merck).....	not determined	acid
Litmus (specially purified).....	not determined	

All were used in approximately 0.2 per cent solution in water, save those which were insufficiently soluble in water at room-temperature and which were used in saturated solution, those of which a 0.2 per cent solution was so deep in color as to be opaque (gentian violet, dahlia, methyl violet B, methylene blue, etc.) in which cases the solutions were diluted to a desirable transparency, those which were insoluble or very sparingly so in water, in which cases either the powder was shaken up directly in the mixture of water and lipoid (Sudan III, Scharlach R) or

TABLE 2.

Name of dye.	IN ACID SOLUTION.		IN ALKALINE SOLUTION.	
	Water.	Ethyl acetate.	Water.	Ethyl acetate.
Iodin-eosin ...	colorless	yellow	red	colorless
Erythrosin	colorless	yellow	red	colorless
Acid fuchsin ...	red	colorless	colorless*	colorless
Rosolic acid. ...	pale yellow	deep yellow	red	pale yellow
Gentian violet.	violet	slightly paler violet	purple	deep orange-red
Crystal violet...	deep blue	pale blue-violet	deep red-violet	pale blue-violet
Methyl violet B	deep purple	light purple	blue-purple	deep orange-red
Methyl green...	green	pale pink	colorless*	colorless
Dahlia	deep violet	red-violet	deep violet	deep orange-red
Rhodamin.....	deep dichroic pink	pink	deep dichroic pink	colorless
Pyronin.....	red-yellow dichroic	faint pink	red-yellow dichroic	faint pink
Thionin	deep blue	colorless	blue	orange
Safranin.....	red	colorless	red	pink
Neutral red. ...	red	colorless	colorless	yellow
Methylene blue	blue	colorless	blue	red
Tropaeolin 000	orange	yellow	red	colorless
Orange G.....	orange	pale yellow	red	colorless
Methyl orange.	orange	very pale yellow	red	colorless
Bordeaux red..	red	yellow	red	colorless
Congo red.....	blue ppt.	colorless	red	colorless
Bismarck brown	deep red	pale yellow	yellow	orange
Sudan III.....	colorless	red	colorless	red
Scharlach R....	colorless	red	colorless	red
Wool black.....	purple	pink-violet	purple	faint pink or colorless
Azure II.....	blue	blue	blue	red
Azure II eosin.	blue	colorless	blue-green dichroic	pink
Picric acid.....	yellow	yellow	yellow	yellow
Lacmoid.....	colorless	pink	blue	colorless
Sulphindigotate of soda.....	blue	colorless	blue	colorless
Haematin	brown ppt.	deep brown	deep brown	very faint yellow
Orcein.....	deep red	red	deep violet	colorless
Carminic acid..	yellow	yellow	red	colorless
Carmine.....	red	colorless	red	colorless
Litmus.....	red	faint pink	blue	colorless

* Methyl green and acid fuchsin in alkaline solution slowly decolorize, but so long as they retain their color they remain in the watery layer and do not enter the ethyl acetate layer. If, after decolorization, acid be added separately to samples of the watery and of the ethyl acetate layers only that from the watery layer gives a color.

else the solution was made in alcohol and diluted with water (safranin, hæmatin). In the case of hæmatin the powder was first dissolved in absolute alcohol and diluted to 20 times its volume with water, forming a suspension which only gradually settled out.

(a) *Experiments with ethyl acetate.* To about 10 cc. of each of the above solutions was added 0.5 cc. of $\frac{N}{10}$ potassium hydrate

or of $\frac{N}{10}$ hydrochloric acid and the resultant solution shaken up in a test-tube with 10 cc. of Kahlbaum's ethyl acetate. In some cases the acid was added first and excess of alkali afterwards or vice versa, in most cases the acid and alkali were added to separate portions each of which was shaken up with ethyl acetate and this latter procedure, especially in the case of certain of the other lipoids experimented with, was found to be the most satisfactory. Ethyl acetate forms a fine suspension in water which, as a rule, rapidly clears up; equilibrium of the dissolved dye between the water and ethyl acetate phases of the suspension is rapidly attained, so that a single vigorous shaking of the test-tube sufficed to bring about a distribution of the dye which was not appreciably altered by further shaking.

The results obtained are given in Table 2 on p. 5.

(b) *Experiments with ethyl butyrate.* The experimental procedure was the same as when ethyl acetate was used, but, inasmuch as the rate of the hydrolysis by hydroxyl ions is more rapid than in the case of ethyl acetate, determinations must be made as rapidly as possible and in order to secure alkaline solutions a considerably greater quantity of alkali had to be added than in the experiments with ethyl acetate, the exact amount depending upon the rapidity with which the two layers separated after shaking up.

The following were the results obtained:

TABLE 3.

Name of dye.	IN ACID SOLUTION.		IN ALKALINE SOLUTION.	
	Water.	Ethyl butyrate.	Water.	Ethyl butyrate.
Erythrosin . . .	colorless	yellow	red	colorless
Acid fuchsin. . .	red	colorless	red*	colorless
Gentian violet.	deep blue-purple	colorless	deep red-purple	lighter red-purple
Methyl green..	green	colorless	green*	colorless
Thionin	blue	colorless	blue	pale violet
Neutral red. . .	red	pale yellow	colorless	yellow
Methylene blue	blue	colorless	blue	colorless
Orange G.	orange	colorless	red	pale yellow
Bismarck brown.	brown	yellow	yellow	yellow
Azure II.	blue	colorless	blue	colorless
Orcein.	pink	brown	violet	pale yellow

* Until the solutions decolorized.

(c) *Experiments with ethyl benzoate.* The experimental procedure was the same as in the previous experiments, but as the suspension formed by shaking is in this case much coarser equilibrium of the dye between the two phases of the suspension is much more slowly attained, and it is necessary to shake very thoroughly; for this reason, also, it is advisable to perform the experiment with acid and alkaline solutions separately, as the dye when once dissolved in the ethyl benzoate is not readily extracted again. As in the case of ethyl butyrate a considerable excess of alkali has to be added to secure an alkaline solution.

The following were the results obtained:

TABLE 4.

Name of dye.	IN ACID SOLUTION.		IN ALKALINE SOLUTION.	
	Water.	Ethyl benzoate.	Water.	Ethyl benzoate.
Erythrosin . . .	colorless	yellow	red	colorless
Acid fuchsin . .	red	colorless	red*	colorless
Rosolic acid . .	colorless	yellow	red	colorless
Methyl green . .	green	colorless	green*	colorless
Neutral red . . .	red	colorless	pale yellow	deep yellow
Methylene blue	blue	colorless	blue	red
Orange G.	orange	colorless	red	colorless
Bismarck				
brown	red-brown	pale yellow	very pale yellow	golden brown
Azure II	blue	colorless	blue	red
Orcein	colorless	golden brown	violet	colorless

* Until the solutions became decolorized.

The ethyl acetate, ethyl butyrate and ethyl benzoate used in the above experiments were Kahlbaum's C. P. The triacetin and triolein used in the experiments described below were very kindly supplied to me by Dr. A. E. Taylor, who had prepared them himself in a pure condition.

(d) *Experiments with triacetin.* The technique was the same as in the previous experiments, only smaller quantities used. To 2 cc. of the dye-solution was added .25 cc. of $\frac{N}{10}$ HCl or KOH and the solution was then shaken up with 2 cc. of triacetin. Equilibrium of the dye between the two phases of the suspension was very slowly attained and it was necessary to shake very thoroughly, and absolutely essential to perform the experiments with acid and alkali separately. Triacetin does not saponify

very rapidly in the presence of alkali, and the emulsion formed by shaking up with water clears up fairly rapidly.

The following were the experimental results obtained:

TABLE 5.

Name of dye.	IN ACID SOLUTION.		IN ALKALINE SOLUTION.	
	Water.	Triacetin.	Water.	Triacetin.
Erythrosin . . .	colorless	yellow	red	colorless
Acid fuchsin. . .	red	colorless	red*	colorless
Rosolic acid. . .	colorless	yellow	red	colorless
Methyl green. . .	green	colorless	green*	violet
Dahlia.	blue	violet	deep red-violet	light red-violet
Pyronin.	red	colorless	deep pink	light pink
Neutral red. . . .	red	colorless	yellow	deep yellow
Methylene blue	blue	colorless	blue	colorless
Orange G.	orange	colorless	red	colorless
Bordeaux red.	red	red-brown	red	colorless
Bismarck brown.	red-brown	light yellow	nearly colorless	golden-brown
Azure II.	blue	colorless	deep blue	pale blue
Azure II eosin.	blue	colorless	blue	colorless
Orcein	colorless	golden-brown	violet	pale pink
Carminic acid. .	orange	deep yellow	red	colorless

*Until decolorized. Even after the methyl green was decolorized; so that the watery layer became colorless, the triacetin layer remained violet.

(e) *Experiments with triolein.* Triolein is not suitable for experiments of this class inasmuch as it is very rapidly hydrolyzed by alkali, and as soon as a small amount of hydrolysis has taken place the emulsion formed on shaking up with water is stable and even if it does partially separate into two layers each layer contains suspended particles of the other phase, and the apparent color of each layer is really an admixture of the colors of both. The same is partially true even for the acid solutions,

TABLE 6.

Name of dye.	IN ACID SOLUTION.		IN ALKALINE SOLUTION.	
	Water.	Triolein.	Water.	Triolein.
Neutral red. . .	red	colorless	colorless	yellow
Azure II.	blue	blue	blue	red
Azure II eosin.	blue	colorless	blue	colorless
Orcein.	deep red	red	blue-violet	colorless or pale pink

inasmuch as the emulsion formed by shaking up triolein with acid water only clears up very slowly, especially in the presence of certain dyes. Such experiments as could be performed, however, led to the same general results as the experiments with the other lipoids.

The results obtained are given in Table 6, p. 8.

(f) *Discussion of the foregoing results.* In reviewing the above results one thing stands out clearly, namely, that the solubility of an acid dye in lipid is increased by the addition of acid while that of a basic dye is increased by the addition of alkali. In other words, the free color-acids or color-bases are more soluble in lipid than their salts. No marked difference exists between the acid and basic dyes in this respect, the free color-acids being about as soluble in lipid as the free color-bases. The *salts* of the basic dyes, however, are as a rule soluble to some extent in lipid, while those of the acid dyes, as a rule, are not. Thus, of the acid dyes only picric acid, rosolic acid and hæmatin are soluble in ethyl acetate in alkaline solution, the two latter only very slightly so; while of the basic dyes gentian violet, crystal violet, methyl violet B, dahlia, rhodamin, Bismarck brown and azure II in acid solution are all markedly soluble in ethyl acetate. Hence the conclusion of Overton's that the basic dyes are much more soluble in lipid than the acid dyes was due to the fact that he, as a rule, confined his observations to the *salts* of the dyes in neutral solution.

It is worthy of note that certain of the basic blue or violet dyes (*i.e.*, gentian violet, methyl violet B, dahlia, thionin, methyl green and methylene blue) give when shaken up with certain of the lipoids in alkaline solution a red, orange, or, in the case of methyl green shaken up with triacetin, a violet lipoidal layer. These colors are due to impurities in the dyes. Thus commercial methylene blue, unless specially purified, is well-known to contain a greater or less admixture of methylene-violet and methylene-azure.¹ By repeated extraction of an alkaline solution with ethyl acetate (adding fresh alkali from time to time to replace that taken up by the ethyl acetate) we can completely

¹ Lee: *Microtometist's Vade-Mecum*, 6th ed., p. 225, 1905; Gustav Mann: *Physiological Histology*, Oxford, p. 405, 1902.

extract the red coloring-matter from the methylene blue and the purified methylene blue thus obtained is not soluble in ethyl acetate either in acid or in alkaline solution; while the red coloring matter which has been thus extracted, upon shaking up the ethyl acetate solution with acid water, goes entirely into the watery layer giving a blue solution, and upon again rendering the solution alkaline it goes *entirely* back in to the ethyl acetate layer, giving a red solution. I have found that the same is true for the orange-red coloring-matter in thionin. As regards the violet coloring-matter which is obtained from an alkaline solution of methyl green by shaking it up with triacetin, this is methyl violet.¹ If the methyl violet be removed from methyl green by repeated extraction of the solution with chloroform the pure solution of methyl green no longer tinges triacetin whether shaken up with it in acid or in alkaline solution.

It should here be noted that methylene violet and methyl violet are both basic. It appeared to me to be of interest to ascertain how far the above facts applied to the actual staining of tissue-elements of a known lipoidal character. Experiments were therefore carried out upon the fat-cells in the omentum and upon red blood corpuscles.

(g) *Experiments upon the fat-cells in the omentum.* Acid and alkaline solutions of the various dyes were made up as follows: To 9 cc. of each of the above-mentioned solutions was added 1 cc. of $\frac{N}{10}$ hydrochloric acid or of $\frac{N}{10}$ potassium hydroxide. Freshly-cut pieces of the omentum of a rabbit were placed in these solutions for about five minutes, washed in $\frac{N}{100}$ hydrochloric acid or potassium hydroxide and examined immediately.

The following were the results obtained:

(i) *With gentian violet.*

(a) In acid solution: Fat-cells colorless, nuclei blue, connective-tissue diffuse blue.

(b) In alkaline solution: Fat-cells colorless, nuclei and connective-tissue diffuse blue.

(ii) *With thionin.*

(a) In acid solution: Fat-cells colorless, nuclei and connective-tissue diffuse blue.

(b) In alkaline solution: Fat-cells violet, nuclei violet.

¹ Gustav Mann: *loc. cit.*, p. 424.

(iii) *With neutral red.*

(a) In acid solution: Fat-cells colorless, nuclei pink.

(b) In alkaline solution: The neutral red is precipitated, the tissue is colorless with the exception of the fat-cells which are a faint yellow. If, however, we stain for 10 minutes in neutral red to which only a little alkali has been added (4 or 5 drops of $\frac{N}{10}$ KOH to 9 cc.), just enough to make the solution a muddy red without precipitating the dye completely, the fat-cells are now stained yellow and the connective-tissue a diffuse yellow, while the fat-cells stained in acid neutral red for a similar length of time remain colorless, although the interstitial tissue and the nuclei of the connective-tissue are stained red. If the tissue which has been stained in alkaline neutral red be now immersed for an instant in acid the dye attached to the nuclei and connective tissue is attacked first and the nuclei are stained pink while the fat-cells remain yellow.(iv) *With methylene blue.*

(a) In acid solution: Fat-cells colorless, connective-tissue diffuse blue.

(b) In alkaline solution: Fat-cells colorless, nuclei, connective-tissue and interstitial tissue deep blue.

(v) *With Bordeaux red.*

(a) In acid solution: Fat-cells colorless, nuclei and connective-tissue red.

(b) In alkaline solution: Fat-cells colorless, connective-tissue diffuse red.

(vi) *With Bismarck brown.*

(a) In acid solution: Fat-cells colorless, nuclei brown, connective-tissue unstained.

(b) In alkaline solution: Fat-cells bright yellow, connective-tissue diffuse light yellow-brown.

(vii) *With Sudan III.*

Both in acid and in alkaline solutions fat-cells deep red.

(viii) *With azure II.*

(a) In acid solution: Fat-cells colorless, nuclei light blue, connective-tissue diffuse light blue.

(b) In alkaline solution: Fat-cells colorless or faint violet, nuclei and connective-tissue deep blue.

(ix) *With orcein.*

(a) In acid solution: Fat-cells colorless or very faint pink, nuclei pink.

(b) In alkaline solution: Entire tissue colorless.

(x) *With carminic acid.*

Both in acid and alkaline solutions, even after an hour's immersion in the solutions, the fat-cells were colorless.

It will be seen that in those cases in which the fat cells stained at all, namely, when thionin, neutral red, Bismarck brown or Sudan III were used, the fat-cells behaved exactly like the ethyl acetate in the experiments tabulated in Table 2, taking up the basic dyes from alkaline solutions rather than from acid solutions and taking up the Sudan III both from acid and alkaline solutions. And, be it noted, those solutions in which the connective tissue and nuclei stain best are not those in which the fat-cells stain most deeply, in fact, there is no correspondence between the depth of staining of the two sets of tissue-elements.

(h) *Experiments with red blood corpuscles.* Cover-glass films of blood (human) were made, dried in air and then placed film side uppermost in a flat dish and a fixing solution poured over them. The fixing-solution was made up as follows:

Mercuric chloride.....	2 grams
Formaldehyde	10 cc.
Absolute alcohol	100 cc.

After one-half hour immersion in this fluid the films were taken out, washed in water and immersed for five minutes in acid and alkaline solutions of the dyes, made up by adding 1 cc. of $\frac{N}{10}$ HCl or KOH to 9 cc. of the 0.2 per cent or saturated solutions of the dyes. The Bordeaux red, however, was used in 1 per cent solution, while the Congo red and tropæolin 000 were used in $\frac{M}{100}$ solution. After immersion in the acid or alkaline staining media the films were washed in $\frac{N}{100}$ HCl or KOH respectively, the superfluous fluid was removed by filter-paper, and the films were dried for one-half hour at a temperature of 60° to 70°. They were then mounted in Canada balsam and examined at once.

The results obtained are given in Table 7.

It will be seen that in all cases in which the red blood corpuscles stained at all they behaved towards the dye in the same way as ethyl acetate does, that is, they take up acid dyes more readily from acid than from alkaline solutions and, with the exception of methylene blue, they take up basic dyes more readily from alkaline solution. But it is perfectly evident that the ability of red blood corpuscles to stain in a dye *cannot be predicted from the lipoid solubility of the dye*, inasmuch as the basic phenyl-methane dyes, some of which are very soluble in lipoid or contain

impurities which are very soluble in lipid, stain the red blood corpuscles only faintly or not at all, while the sulphonic acids of the azo group (tropæolin 000 and orange G) which, even in acid solution, are sparingly soluble in lipid stain the red blood corpuscles deeply.

Fearing that the use of mercuric chloride might have obscured the results, since the heavy metals render tissues more basic,¹ I repeated these experiments with films simply fixed in formal-

TABLE 7.

Name of dye.	COLOR OF RED BLOOD CORPUSCLES STAINED IN—	
	Acid solution.	Alkaline solution.
Erythrosin.....	pale yellow or colorless	colorless
Acid fuchsin.....	deep red	colorless
Gentian violet.....	colorless	colorless
Crystal violet.....	colorless	colorless
Methyl violet B.....	colorless	colorless
Methyl green.....	colorless	colorless
Dahlia.....	colorless, some violet	colorless
Pyronin.....	very faint pink	colorless
Thionin.....	pale blue to colorless	colorless
Neutral red.....	colorless	pale yellow
Methylene blue.....	blue	colorless
Tropæolin 000.....	deep golden-yellow	colorless or very pale yellow
Orange G.....	deep golden-yellow	colorless or very pale yellow
Bordeaux red.....	deep red	colorless
Congo red.....	colorless or very pale brownish	colorless
Bismarck brown.....	light yellow-brown	colorless or very pale yellow
Azure II.....	colorless	light blue
Azure II eosin.....	colorless	colorless
Orcein.....	colorless	colorless
Carminic acid.....	pink	colorless

dehyde-alcohol. The fixation was not good, but such results as could be obtained were identical with those tabulated above.

(j) *The effect of proteins upon the distribution of dyes in acid and alkaline solutions between ethyl acetate and water.* The proteins used were neutral potassium caseinate, gelatin and protamin sulphate; casein, gelatin and protamin being chosen as examples

¹ T. Brailsford Robertson: this *Journal*, i, p. 279, 1906.

of proteins which are, respectively, predominantly acid,¹ neutral² and basic.³ All were used in 0.2 per cent solution. The procedure was as follows: In each of 8 test-tubes were placed 5 cc. of the solution (0.2 per cent or saturated) of the dye under investigation. In two of these were placed 5 cc. of water, in two others 5 cc. of the casein solution, in two others 5 cc. of the gelatin solution and in the two remaining tubes 5 cc. of the protamin solution. Then to one of each of these pairs of tubes was added $\frac{1}{2}$ cc. of $\frac{N}{10}$ HCl and to the other $\frac{1}{2}$ cc. of $\frac{N}{10}$ KOH. In each tube were now placed 10 cc. of ethyl acetate and the tubes were shaken vigorously. It should be mentioned that while the potassium caseinate and gelatin solutions had been freshly made up, the protamin sulphate (in 1 per cent solution) had been standing for some time (under sterile conditions) and consequently must have contained a certain percentage of arginin sulphate.⁴ The results obtained are shown in Table 8.

Remarks on the Results Tabulated.

Gentian violet: Alkaline watery layers all clear blue-purple, the blueness of the purple decreases in the following order: Casein solution, gelatin solution, protamin solution, water.

Crystal violet: The alkaline emulsions containing gelatin and protamin cleared quickly; the emulsion containing no protein cleared very slowly.

Dahlia: The alkaline emulsion containing protamin separated into two layers very quickly, the others very much more slowly. It is not usually recognized that the addition of a colloid may, under certain conditions, render an *emulsion* less instead of more stable.

Pyronin: The watery layers are all red by transmitted light and muddy yellow by reflected light; with the exception of the alkaline solution containing protamin in which the yellow seen by reflected light is much diminished, so that the solution looks redder than the others.

Methylene blue: The concentration of the solution used was 0.4 per cent.

¹ Van Slyke and Hart: *Amer. Chem. Journ.*, xxxiii, p. 461, 1905; T. Brailsford Robertson: this *Journal*, ii, p. 317, 1907.

² W. B. Hardy: *Journ. of Physiol.*, xxiv, p. 288, 1899; T. Brailsford Robertson: *Journ. of Phys. Chem.*, x, p. 524, 1906; xi, p. 437, 1907 (on the general significance of the isoelectric point).

³ F. Miescher, quoted after Gustav Mann: *Chemistry of the Proteids*, p. 419, 1906; A. E. Taylor: *Univ. of Calif. Publ., Pathol.*, i, p. 7, 1904.

⁴ A. E. Taylor: *loc. cit.*

Bismarck brown: The alkaline emulsion containing casein separates into two layers much more slowly than the others.

Azure II eosin: All the alkaline watery layers were violet by transmitted light and green by reflected light with the exception of that containing protamin which was light blue by transmitted or reflected light. The acid watery layers were all blue, those containing protein being, however, darker, that containing protamin darkest of all. Further addition of acid to the watery layer not containing protein did not darken its color.

These experiments show clearly that in many cases the dye combines chemically with protein. Not only are the colors of solutions of the dyes containing protein frequently different from the colors of solutions in water (erythrosin, protamin; acid fuchsin, protamin; gentian violet, casein, gelatin, protamin; azure II eosin, protamin, gelatin, casein; carminic acid, protamin), but the proteins are frequently precipitated by the dyes (acid fuchsin, casein, gelatin; crystal violet, gelatin; orange G, casein, protamin; carminic acid, protamin) and the dye is frequently partially or completely held back in the watery layer when otherwise it would pass into the ethyl acetate layer (erythrosin, casein; gentian violet, casein, gelatin; neutral red, casein; Bismarck brown, casein, gelatin). These facts can only be interpreted as indicating chemical combination between the dyes and protein.¹

(k) *Application of certain of the above results to the use of indicators.* If to 10 cc. of a concentrated (0.2 per cent) and very faintly acid solution of neutral red we add a single drop of $\frac{N}{10}$ KOH the color of the solution does not perceptibly change, nevertheless a great change in respect to the lipid-solubility of the neutral red is seen if we shake up the original and the alkaline solution with ethyl acetate. On shaking up with the faintly acid solution the ethyl acetate remains absolutely colorless, while on shaking up with the faintly alkaline solution the ethyl acetate layer is stained deep yellow. In two ways the indicator is rendered more sensitive by this method; in the first place a trace of the yellow modification of neutral red, which would be invisible in watery solution owing to the great excess of the red modification,

¹ Compare Berthelot et Jungfleisch: *loc. cit.*; and Berthelot: *loc. cit.*; Nernst: *Theoretical Chemistry*, Trans. of 4th ed., p. 487, 1904.

is removed by the ethyl acetate and thereby rendered visible. In the second place let us suppose that the coefficient of distribution, $\frac{\text{concentration of lipid layer}}{\text{concentration of watery layer}}$, is 100 : 1 for the yellow modification of neutral red and zero for the red modification. Then at any given concentration b of hydroxyl ions, if y be the concentration of the red modification and x that of the yellow modification:

$$x = kf(b) y$$

where k is a constant. Now let this solution be shaken up with ethyl acetate and let the concentration of the yellow modification in the watery layer now be x_1 , while that of the red modification is y_1 , and that of the yellow modification in the lipid layer is x_2 , then we have:

$$\begin{aligned} x_1 &= kf(b) y_1 \\ x_2 &= 100 x_1 \\ x_2 &= 100 kf(b) y_1 \end{aligned}$$

that is, the concentration of the yellow modification in the lipid layer is 100 times its concentration in the watery layer and, provided $f(b)$ were a linear function, it would be the same as would be produced in the watery layer by 100 times the concentration of hydroxyl ions. In other words, what we may define as the "sensitiveness" of the indicator, namely, the concentration of the new modification of the indicator produced by a given excess of hydrogen or hydroxyl ions is, if we use another solvent immiscible with water and compare the two layers, multiplied by the distribution-coefficient between the two solvents of the new modification of the indicator. In addition to this, as I have said, we have an apparent or "physiological" increase in the sensitiveness of the indicator, due to the physical separation of the two colors; a separation which has hitherto been secured, partially or completely, by the use of specially colored screens or lights,¹ by the addition of other coloring matters,² or by the use of the spectrophotometer.

¹ Duprè, quoted after Sutton: *Volumetric Analysis*, 9th. ed., p. 142, 1904.

² Förster, quoted after Sutton: *ibid.*, p. 36.

III. GENERAL CONSIDERATIONS.

At this date, after the classic researches of Ehrlich¹ and the decisive results obtained by Mathews and Heidenhain,² it is indeed strange to find the phenomena of protoplasmic staining by dyes alluded to, simply, as phenomena of "solid solution."³ To recount here the arguments advanced and the results obtained by these authors would be superfluous, since they are neither inaccessible nor unknown, but were any additional argument required in support of the chemical theory of staining the results described in this paper afford a powerful one. In the first place we have seen that there is no correspondence whatever between the depth of staining of connective-tissue cells and that of fat-cells in the same tissue. If the staining of the connective-tissue cells merely depended upon the solubility of the dye in a superficial lipoid layer and the staining of the tissues were merely an instance of "solid solution" then all those substances which stain the connective tissue cells should also stain the fat-cells and vice versa. "Specific" stains should also be impossible on the basis of Overton's hypothesis; different tissue elements might conceivably take up the stain to different extents, but the order of the depth of staining of different elements should be identical whatever the stain used, and it would be impossible to stain one element deeply to the total or partial exclusion of the rest with one stain and with another stain to stain a different element to the exclusion of the former element. The staining of nerve-endings by methylene blue, of elastic tissue by orcein, of leucocytes and blood-platelets by iodine-eosin,⁴ of mucous cells by Hoyer's method⁵ and of smooth muscle by Unna's method,⁶ only to mention a few among a host of examples, emphatic-

¹ P. Ehrlich: *Histologie und Klinik des Blutes*, Berlin, 1891.

² A. Mathews: *Amer. Journ. of Physiol.*, i, p. 445, 1898; M. Heidenhain: *Arch. f. d. ges. Physiol.*, xc, p. 115, 1902.

³ Overton: *Jahrbücher f. wiss. Botanik*, xxxiv, p. 700, 1900; Höber: *Physikalische Chemie der Zelle und der Gewebe*, 2 Aufl., p. 174.

⁴ Ehrlich and Lazarus: *Die Anämie*, Wien, 1898, quoted after Gustav Mann, *Physiological Histology*, Oxford, p. 214, 1902.

⁵ Hoyer: *Arch. f. mik. Anat.*, xxxvi, p. 310, 1890.

⁶ Unna: *Monatsschr. f. prakt. Dermatol.*, xix, p. 533, 1894; *Zeitschr. f. wiss. Mik.*, xii, p. 243, 1895.

ally contradict this point of view. Moreover, the experiments described above on the influence of protein upon the distribution-coefficient of dyes between lipoids and water indicate clearly chemical combination between protein and certain of the dyes, and the formation of such compounds has been demonstrated in a variety of ways and between a variety of dyes and proteins.¹ Nevertheless we are asked to believe that although these reactions take place *in vitro*, yet the staining of the living cell is unaccompanied by these reactions and is merely a phenomenon of "solid solution." The experiments described above on the staining of red blood corpuscles show clearly the influence of chemical factors. Despite the fact that the red blood corpuscles consist very largely of fat those dyes which are most soluble in fat do not stain them (certain of the basic dyes of the phenyl-methane group), while other dyes which are only sparingly soluble in fat (tropæolin 000, orange G) stain them deeply. I consider it probable that most, if not all, of the dyes *enter* the corpuscles but that only certain, and particularly acid, dyes combine with some constituent of the corpuscles. If one inquires upon what data Overton bases his belief that the staining of tissues is a phenomenon of solid solution, we find that it is based upon the single (and erroneous) premise that the ability of dyes to stain living cells is simply proportional to the solubility of the dye in lipid. Such an argument, if justifiable in one instance, must be equally valid in a similar instance, and in the case of tetanus toxin, for example, the action of which is almost wholly confined to the nervous system on account of its high solubility in lipoids.² We must suppose that its action is wholly due to its forming a "solid solution" in the lipoids of the nervous system and not at all to any chemical action; I presume that the most enthusiastic adherent of the solid solution hypothesis would scarcely venture to subscribe to such an opinion, yet it is merely an extreme example of the general type of argument employed by Overton in reference to the action of dyes and of the so-called "indifferent" narcotics upon living tissues. As Loeb has pointed out "the

¹ A. P. Mathews: *loc. cit.*; M. Heidenhain: *loc. cit.*; Rosenbloom and Gies: *Proc. Amer. Soc. Biol. Chem.*, this *Journal*, iii, p. xxxix, 1907.

² Hans Meyer and F. Ransom: *Arch. f. exper. Path. u. Pharm.*, xlix, p. 369, 1902.

rapidity of the absorption of narcotics may be due to their solubility in oil and yet the effect they produce may be due to something entirely different."¹

It is therefore clear that the theory of a lipid limiting membrane at the surface of living cells, in conjunction with a "solid solution" theory of staining is an untenable one. It remains to be considered whether, in any case and without excluding the possibility of chemical combination between dyes and tissue elements, the theory of a lipid layer at the limiting surface of cells can be retained.

If we examine the data upon which the theory of the lipid membrane has been founded, we find that they resolve themselves into three classes as follow:

(1) The phenomenon of "rounding up" of droplets of protoplasm when squeezed out of cells into a watery liquid.²

(2) The relation which is stated to exist between the lipid solubility of a narcotic, poison or dye, etc., and its effect upon living matter.³

(3) The relation which is stated to exist between the osmotic pressure of a solution and its toxic or plasmolytic action, demanding the existence at the surface of the cell of a semipermeable membrane.⁴

We will deal with each of these arguments seriatim.

(1) *The phenomenon of the rounding up of droplets of protoplasm in watery liquids.*

It has been assumed, following Quincke, that this phenomenon necessitates the assumption that at the surface of the droplet an oily layer is formed. Nevertheless this assumption is by no

¹ J. Loeb: *Dynamics of Living Matter*, p. 41, 1906.

² Quincke: *Sitzungsber. d. Berl. Akad. d. Wiss.*, p. 791, 1888.

³ Overton: *Jahrbücher f. wiss. Botanik*, xxxiv, p. 669, 1900; *Studien über die Narkose*, Jena, 1901.

⁴ de Vries: *Jahrbücher f. wiss. Botanik*, xiv, p. 27, 1884; *Zeitschr. f. physikal. Chem.*, ii, p. 415, 1888; iii, p. 103, 1889; Traube: *Arch. f. Anat. u. Physiol.*, Physiol. Abt., pp. 87 and 129, 1867; Hamburger: *Arch. f. Anat. u. Physiol.*, Physiol. Abt., p. 476, 1886; p. 31, 1887; *Zeitschr. f. physikal. Chem.*, vi, p. 319, 1890; *Zeitschr. f. Biol.*, xxvi, p. 414, 1889; Pfeffer: *Pflanzenphysiologie*, 2 Aufl., p. 91, 1897; Overton: *Vierteljahrsschrift der Naturforschergesellschaft*, Zurich, p. 159, 1895; p. 88, 1899.

means justified, for there are many other well-known alternatives. Ramsden has shown that solid or highly viscous films are very rapidly formed at the surface of protein solutions¹ and upon mechanical agitation these films may be broken and fresh ones formed. Hence it follows that proteins can be coagulated by mere mechanical agitation² or by the purely surface-action of fine powders such as burnt clay or charcoal³ or the pores of certain filters. The formation of this film is due to the diminution in surface-tension caused thereby and the consequent migration to the surface of the dissolved particles.⁴ Gibbs has pointed out that such a film constitutes a separate phase with its own characteristic equation. It is evident that the physical agency which leads to the assumption and retention of the globular form by protoplasmic droplets may be the formation of such films at the surface of the droplet. Such films are very stable; if one shakes up ethyl benzoate with water in the presence of colloids such as certain of the dyes (especially erythrosin, methylene blue and Bismarck brown; of these Bismarck brown shows the phenomenon in the most marked degree) an emulsion is formed which clears up more or less slowly; eventually, however, on standing, large drops of the benzoate are formed in the water layer which remain attached to the sides of the test-tube. These drops are surrounded by membranes; if one now gently shakes the tube the membranes break, setting free the drops of ethyl benzoate which now fall to the bottom of the tube. The membranes, however, remain behind attached to the walls of the tube and, on gentle agitation, can clearly be seen floating in the water; they do not redissolve, or only very slowly. This phenomenon

¹ Ramsden: *Zeitschr. f. physikal. Chem.*, xlvii, p. 336, 1904.

² Berthold: *Studien über Protoplasma-mechanik*, 1886, quoted after Gustav Mann, *Chemistry of the Proteids*, p. 274, 1906; Ramsden: *Arch. f. Anat. u. Physiol.*, Physiol. Abt., p. 517, 1894.

³ Hermann: *Arch. f. d. ges. Physiol.*, xxvi, p. 442, 1881.

⁴ J. Willard Gibbs: *Equilibrium of Heterogeneous Substances*, *Trans. Connecticut Acad. of Sciences*, 1878, reprinted in *The Scientific Papers of Willard Gibbs*, i, p. 219, etc., 1906; Rayleigh: *Scientific Papers*, iii, p. 351, 1902; F. G. Donnan: *Zeitschr. f. physikal. Chem.* xxxi, p. 42, 1899. See also W. P. Dreaper: *Journ. Soc. Dyer's Colorists*, xxiii, p. 188, quoted after *Chemical Abstracts*, i, p. 2302, 1907, for interesting examples of the concentration of a solute at surfaces of the solution,

is independent of the relative solubilities of the dye in the two phases of the emulsion and may be seen in cases (for instance in the case of Bismarck brown) where the dye is soluble in both phases.

If chloroform be shaken up with casein, gelatin or protamin solutions it settles to the bottom in fine particles or droplets, which, if numerous, form a milky layer at the bottom of the vessel; by transmitted light, however, they appear perfectly clear. These droplets are extraordinarily stable and do not coalesce however long they stand in contact; they may be repeatedly washed in water until all traces of protein have been removed from the supernatant fluid and they still remain perfectly stable and distinct from one another; they may be shaken up in chloroform or treated with $\frac{N}{10}$ potassium hydroxide without impairing their form or stability. If, however, they be heated to nearly the boiling-point of chloroform under a layer of water the droplets burst and coalesce, forming a homogeneous layer of chloroform. If treated with alcohol they immediately dissolve leaving a fine membranous precipitate floating in the water. Thus if we shake up chloroform with about twice its volume of 1 per cent protamin sulphate and, after standing separate the chloroform droplets by pouring off the supernatant liquid, and then, after repeatedly washing the droplets in water by decantation, add to the small amount of supernatant water about an equal volume of alcohol and gently agitate, the droplets which are thus stirred up into the alcohol-water layer can be seen to rapidly swell up and burst and then the fine membranes which surrounded them can be seen falling down through the alcohol-water. If we now add several volumes of alcohol and shake up the liquid the chloroform droplets all disappear and what we now have is a clear homogeneous solution in which innumerable minute membranes can be clearly seen floating.

These experiments show conclusively that two portions of the same solvent can remain perfectly distinct from one another when separated by a surface-membrane forming a separate phase, and, be it noted, these droplets, even when suspended in chloroform, are spherical.

That these surface films possess quite different properties from the protein in the body of the fluid is evident. In the first

place they are much more insoluble, in the second place "when colloidal mixtures separate to form conjugates a thin layer next the surface remains homogeneous"¹ whereas the body of the fluid becomes heterogeneous. The fact that they are not readily acted on by dilute acid and alkali renders it probable that they are chemically more inert than the protein in the body of the fluid. We know that alterations in chemical equilibria occur in such surface-layers;² it seems probable that owing to this and to the increase in concentration of the protein at the limiting surface polymeric modifications are produced³ and that this process, owing to the resistance to internal molecular motion afforded by the viscosity of such a system, is only partially or very slowly reversible.

That similar considerations hold good when water or substances containing water as solvent are similarly enveloped in a concentration-film is at once evident from the observations of Hardy.

"When 13.5 grammes of dry gelatine are dissolved in 100 cc. of a mixture of equal volumes of absolute alcohol and water, a system is produced which is clear and homogeneous at temperatures above 20°. As the temperature falls below this limit a clouding occurs, which I find to be due to the appearance of fluid droplets which gradually increase in size until they measure 3 μ . On cooling further these fluid droplets become solid and they begin to adhere to one another. In this way a framework is built up composed of spherical masses hanging together in linear rows which anastomose with one another. The frame work is therefore an open structure which holds the fluid phase in its interstices." By experiments on agar it was shown that this interstitial fluid is a *dilute* solution of the colloid. "When once formed the phases have considerable stability. If the droplets are composed of a solid solution one may, by the addition of water, cause them to increase to relatively vast dimensions without their being destroyed; as they increase in size their refractive index

¹ W. B. Hardy: *Journ. of Physiol.*, xxiv, p. 158, 1899; Gustav Mann: *Physiological Histology*, Oxford, p. 123, 1902.

² J. Willard Gibbs: *loc. cit.*; Nernst: *Theoretical Chemistry*, English trans. of 4th ed., p. 664, 1904; Liebreich: *Zeitschr. f. physikal. Chem.*, v, p. 529, 1890; vii, p. 83, 1891; Hulett: *Zeitschr. f. physikal. Chem.*, xxxvii, p. 385, 1901.

³ T. Brailsford Robertson: *Journ. of Physical Chem.*, x, p. 524, 1906; xi, p. 437, 1907. See also William Sutherland: *Proc. Roy. Soc.*, lxxix, B, p. 130, 1907.

approximates more and more to that of the external phase until they finally are lost sight of. The addition of alcohol, however, once more brings them into view and causes them to shrink. Owing to this stability once a configuration has been established one has to far overstep the conditions of its formation in order to destroy it. This would account for the remarkable hysteresis observed in reversible gels. Thus a 10 per cent solution of gelatine in water sets at 21° and melts again at 29.6° and solutions of agar in water set at temperatures about 35° and melt at temperatures about 90° . Similarly with the ternary mixtures. In one holding about 35 per cent gelatine the internal and external phases separate at 20° but they mix again only at 65° . When water is added to a ternary mixture so as to considerably swell the droplets the system is unstable, and the two phases mix at once when it is mechanically agitated."¹

These experiments demonstrate that *spherical* droplets of concentrated protein solutions can retain their integrity for a considerable time when suspended in water, indefinitely when suspended in a solution of the protein of a certain dilute concentration. No "lipoid membrane" is required and no impermeability to substances not soluble in lipid need be assumed. The mechanism which conditions this stability of form is most probably a membrane of the type described above; such a membrane, when nearly dissolved, would readily be broken by mechanical agitation, as in the case of the ternary mixture quoted above. That such membranes exhibit hysteresis is evident from the insolubility of the "mechanical coagula" of Ramsden. That such hysteresis is a common phenomenon where large molecule-complexes of protein are concerned is evident from the following considerations. Normal protein, in solution, is in an unstable condition, reactions spontaneously taking place in the direction $HXXOH + H_2O \rightarrow HXOH + HXOH^2$, and these reactions can be accelerated by enzymes. On heating most proteins, however, the equilibrium (of the higher complexes) is shifted in the direction $HXXOH + HXXOH \rightarrow HXXXXXOH + H_2O$ and coagula are formed. These coagula, on returning to the normal temperature, do not generally redissolve but are relatively stable and with difficulty attacked by enzymes; in fact, only in one case

¹ W. B. Hardy: *Journ. of Physical Chem.*, iv, pp. 255 and 259, 1900.

² A. E. Taylor: *Univ. of Calif. Publ., Pathol.*, i, p. 49, 1904; T. Brailsford Robertson: *this Journal*, ii, p. 344, 1907; *Journ. of Physical Chem.*, x, p. 524, 1906; xi, p. 437, 1907.

has a reversion of heat-coagulation been observed, namely, in the case of the caseinates of the alkaline earths;¹ yet these heat-coagulated proteins must be still further removed from the condition of equilibrium than the normal proteins and their relative stability is merely an example of the hysteresis of large protein molecule-complexes, due probably to excessive internal molecular friction. The "mechanical coagula" of Ramsden are intermediate in character between normal, soluble proteins and heat-coagulated proteins; they resemble fibrin in their general properties but can be rendered still more insoluble by heating.² It appears probable that such temporarily irreversible protein membranes may be in every instance formed at the surface of cells.

We therefore see that the assumption of an oily layer at the surface of cells or of protoplasmic droplets is quite gratuitous in so far as the maintenance of their integrity is concerned, and that the properties of concentration-films of protein quite suffice to account for the phenomena observed.

(2) *The relation between the lipoid-solubility of a narcotic, poison, or dye and its effect upon living matter.*

From the experiments described in this paper it is evident that the facts do not support the hypothesis that there is a direct relation between the lipoid-solubility of a dye and its staining power, on the contrary, there are many facts in direct contradiction to it.

The "vital stains," that is, the dyes which stain living protoplasm or certain elements in the protoplasm most deeply are neutral red, methylene blue, toluidin blue, thionin, Nile blue and safranin,³ to which list methyl green may be added. But of these we have seen that methylene blue, thionin and methyl green are absolutely insoluble in lipoids, although the impurities which are generally associated with these dyes may be, under certain circumstances, soluble in lipid. Overton states that

¹ W. A. Osborne: *Journ. of Physiol.*, xxvii, p. 398, 1901.

² Ramsden: *loc. cit.*

³ Höber: *Physikalische Chemie der Zelle und der Gewebe*, 2 Aufl., p. 172, 1906.

methyl green is soluble in olive oil,¹ but I venture to suggest that the substance which, in his experiments, stained the olive oil was not methyl green but methyl violet. I have shaken up methyl green with olive oil in acid, neutral and faintly alkaline solution and in the neutral and alkaline solutions the olive oil is tinged violet. If, however, we previously extract all the methyl violet from the methyl green solution by means of chloroform, the purified methyl green, whether in acid, neutral, or alkaline solution, communicates no color whatever to the olive oil when shaken up with it. Now methyl green is an excellent stain for living cells, especially staining the nuclei deep green.² Since the color which is imparted to the cell and to the nuclei is green and not violet it is evident that the coloring matter which enters the cell is methyl green and not the trace of methyl violet also present in the solution of the dye. In order, however, to make sure of this point I extracted a solution of methyl green (0.2 per cent) with chloroform until no further color was imparted to the chloroform, the watery layer was then separated off by means of a separating-funnel and boiled for several minutes to expel the dissolved chloroform; this solution, after cooling, imparted no color either to chloroform or to olive oil. On staining living *Paramœcia* and fresh pieces of rabbit's mesentery with this solution the following phenomena were observed: the *Paramœcia* were stained a diffuse light green, the granules of the endoplasm being stained much more deeply than the ectoplasm, the nucleus was not differentiated. On transferring the *Paramœcia* to tap-water and killing them by the addition of a trace of copper salts the nuclei appeared a deep green leaving the remainder of the tissue almost colorless, showing that after death the nuclei took up the stain from the surrounding endo- and exoplasm. The connective-tissue of the rabbit's mesentery was stained a diffuse green, the nuclei of the cells deep green within five minutes. Now in order to stain the nucleus the methyl green must traverse both the external limiting membrane and

¹ Overton: *Jahrbücher f. wiss. Botanik*, xxxiv, p. 669, 1900.

² For our purpose it is immaterial whether the methyl green actually stains the *chromatin* of the living nucleus or not (vide Lee, *loc. cit.*, p. 158), the question at issue being whether the dye enters the cell at all and not whether it stains any given element.

the limiting membrane of the nucleus, hence neither of these can be exclusively lipoidal in composition. Similar arguments apply to methylene blue and thionin.

Since the impurity in methylene blue which is soluble in lipid is blue in acid although it is red in alkaline solution, it might be argued that the substance which really enters the cell when stained in methylene blue is the impurity which is soluble in lipid, the cell-contents acting as acids. This very improbable hypothesis is refuted by the following experiment. Fresh pieces of rabbit's mesentery were stained for five minutes in a 0.4 per cent solution of methylene blue; after thoroughly washing in water so as to free the tissue from adherent dye one piece was examined immediately, another was soaked in $\frac{N}{10}$ potassium hydroxide for several minutes, a third was soaked in ethyl acetate which had been thoroughly shaken up with $\frac{N}{10}$ potassium hydroxide immediately before performing the experiment. If the dye which enters the cells were the lipid-soluble impurity it should be turned red by alkaline water and should be extracted by alkaline ethyl acetate, giving a red solution. On the contrary, however, although a certain amount of the dye was extracted by the alkaline water, none at all or only a barely perceptible amount was extracted by the alkaline ethyl acetate, while, on examination, all three pieces of tissue were found to be about equally stained deep blue, although in the specimens which had been washed only in neutral or alkaline water there was present a faint tinge of violet which was absent in the specimen which had been soaked in ethyl acetate. In all three specimens the nuclei of the connective-tissue cells, as well as the connective tissue fibers were stained an intense blue.

Congo red has been successfully used as a "vital stain,"¹ yet Congo red, whether in acid, neutral, or alkaline solution is insoluble in lipid (ethyl acetate, and, in neutral solutions, ethyl benzoate and olive oil; shaken up in water with the two latter lipoids a very stable, fine emulsion is formed accompanied by membrane-formation such as I have described. If, however, we spread the emulsion out in a thin layer on a microscope-slide it can be clearly seen that the dye is held in minute drop-

¹ Lee: *loc. cit.*, p. 158.

lets of water and that the larger interspaces of lipid are quite unstained).

Sulphindigotate of soda is absolutely insoluble in lipid (ethyl acetate, olive oil), yet it freely passes through the living epithelium of the renal tubules and stains the cells.¹

Thus we see that there is no correspondence between the lipid solubility of dyes and the ease with which they enter living cells. If, regardless of the fact that organisms will live in solutions of the "vital stains" for long periods, if not indefinitely, in an apparently uninjured condition,² we consider all those dyes which enter living cells as toxic, so that they alter and render more permeable their enveloping lipid membrane, the same argument could be applied to all cases of lack of parallelism between lipid solubility and physiological or pharmacological action, the lipid membrane would lose all physiological significance and it would be tantamount to abandoning the theory altogether.

I have elsewhere shown³ that previous treatment with "neutral salts" greatly modifies the power of methyl green or of iodine-eosin to stain *Paramœcia*. In some instances (sodium butyrate, methyl green; magnesium chloride, iodine-eosin) the stainability of the infusoria is greatly reduced. On the basis of Overton's hypothesis it is difficult to see how this can be explained. The assumed lipid membrane being already impermeable to dyes which are not lipid-soluble cannot well be rendered *less* permeable by the action of a salt. At the same time those salts which render *Paramœcia* less stainable in methyl green render them more stainable in iodine-eosin and vice versa, so that if we attributed these results to alterations in permeability of the lipid membrane we should have to suppose that those salts which render the cell-membrane more permeable to methyl green render it at the same time less permeable to iodine eosin and vice versa, an entirely gratuitous assumption inasmuch as the influence of the salts upon the stainability of *Paramœcia* in these dyes

¹ Chrzonszczewsky: *Virchow's Archiv*, xxxv, p. 158, 1866; R. Heidenhain: *Hermann's Handbuch der Physiol.*, v, p. 345; cf. also Gantrelet and Gravellet: *Compt. rend. de l'Acad. des. sci.*, cxliv, p. 1467, 1907.

² A. M. Przesmycki: *Biologisches Centralblatt*, xvii, pp. 321 and 353, 1897.

³ T. Brailsford Robertson: this *Journal*, i, p. 279, 1906.

bears no relation to the acidity or alkalinity of the medium. Similar considerations were found to hold good for the influence of salts upon the toxicity of a variety of alkaloids for *Paramoecium*, *Tubifex* and *Gammarus*.¹ These results, moreover, can only be explained by supposing that the salts enter and chemically alter some constituents of the cell despite their very general insolubility in lipoids. Thus the addition of $\frac{M}{13800}$ strychnin nitrate to a $\frac{N}{52}$ solution of sodium sulphate *diminishes* the toxicity of the sodium sulphate for *Gammarus*; on the basis of Overton's hypothesis we can only conclude that the strychnin *diminishes* the permeability of the lipid membrane for the sodium sulphate; if this were its sole action it should diminish the toxicity of every salt, whereas it *increases* the toxicities of magnesium sulphate, sodium chloride, barium chloride and sodium acetate, hence, in addition to its action in diminishing the permeability of the lipid membrane for salts it must exert its own separate toxic action. This separate toxicity of strychnin nitrate could not possibly be *greater* than the toxicity of sodium sulphate alone, otherwise the addition of strychnin nitrate could not *diminish* the toxicity of sodium sulphate; hence the additional toxicity conferred upon a salt by the addition to it of $\frac{M}{13800}$ strychnin nitrate could not be greater than the toxicity of sodium sulphate alone. But the additional toxicity conferred upon $\frac{N}{52}$ sodium acetate by the addition of $\frac{M}{13800}$ strychnin nitrate is over three times the toxicity of sodium sulphate alone, hence we should have to conclude that in this instance some additional toxic substance was entering the cell as well as strychnin nitrate, that is, we should have to suppose that the strychnin *increases* the permeability of the lipid membrane for sodium acetate. Thus we would arrive at the conclusion that strychnin nitrate at the same time renders the membrane *less* permeable for one salt and *more* permeable for another salt, a conclusion which would necessitate our endowing the lipid membrane with very peculiar and entirely hypothetical properties.

Hence we see that the supposed relation between the lipid solubility of a narcotic, poison, dye or salt and its effect upon living matter is one which will not bear a critical examination,

¹ T. Brailsford Robertson: This *Journal*, i, p. 507, 1906.

which certainly does not hold good for dyes and which probably, therefore, does not hold good for the majority of substances which may exert an influence upon living matter.

- (3) *The relation which is stated to exist between the osmotic pressure of a solution and its toxic or plasmolytic action, demanding the existence at the surface of the cell of a semipermeable membrane.*

That the influence of the concentration of a medium upon the cells or organisms suspended in it cannot be attributed to purely osmotic factors depending upon the presence of a semipermeable membrane at the surface of living cells has been shown more especially by the experiments of Loeb,¹ Wolfgang Ostwald,² and Osterhout.³ Loeb, experimenting with a marine crustacean (*Gammarus*) found that they died rapidly in distilled water, but that they died equally rapidly in solutions of cane sugar, dextrose, or even sodium chloride which were isotonic with sea-water. They died even more rapidly in a solution containing all the salts in sea-water, with the exception of sodium chloride, in the concentration in which they occur in sea-water. If, now, to the highly toxic solution of sodium chloride in the concentration in which it occurs in sea-water there be added the even more toxic combination of the remaining salts in sea-water in the concentration in which they occur therein, we obtain a solution in which *Gammarus* lives as long as it does in sea-water, that is, as far as our purposes are concerned, indefinitely.

If the action of salts were a simply osmotic one the relation between concentration and toxicity should be simply a linear one. Wolfgang Ostwald, however, working with fresh-water *Gammarus*, found a sudden and extraordinary increase in toxicity at a critical concentration of all the solutions the effects of which he investigated, and this sudden increase in toxicity did not by any means occur at the same osmotic pressure in different solutions. Osterhout, working with a fresh-water alga (*Vau-*

¹ J. Loeb: *Arch. f. d. ges. Physiol.*, xcvi, p. 394, 1903; ci, p. 340, 1904.

² Wolfgang Ostwald: *ibid.*; cvi, p. 568, 1905; *Univ. of Calif. Publ., Physiol.*, ii, p. 163, 1905.

³ W. J. V. Osterhout: *This Journal*, i, p. 363, 1906; *Univ. of Calif. Publ., Botany*, ii, pp. 227, 229, 231 and 235, 1906; *Botanical Gazette*, xlii, p. 127, 1906; xlii, p. 259, 1907.

cheria sessilis), found that it lived three or four weeks in distilled water and indefinitely in a dilute sea-water corresponding in osmotic pressure to about a $\frac{3}{32}$ molecular sodium chloride. But it was killed in a few minutes in pure $\frac{3}{32}$ molecular sodium chloride and in a few days by ten thousandth molecular sodium chloride. Numerous similar and equally striking experiments of Osterhout's, an account of which will be found in the papers to which I have alluded, all point to similar conclusions. Indeed the whole series of experiments upon antagonistic salt-effects and physiologically balanced salt-solutions¹ establish beyond doubt the very subordinate rôle played by osmotic pressure in phenomena of toxicity.

We thus see that all three lines of argument which have been advanced in support of the theory of a lipid limiting membrane at the surface of living cells are either fallacious or admit of an alternative explanation. Deprived of all foundation in fact, therefore, and in direct contradiction to many known phenomena, the theory of a continuous lipid membrane at the limiting surface of cells must necessarily be abandoned. On the other hand the known properties of proteins lead us to suspect that at the surface of every living cell there must exist a thin, optically homogeneous, sparingly soluble protein membrane. It is not impossible that, lying under this membrane, there exists a more or less closely packed assemblage of fatty particles held in the meshes of the protoplasm. That the medullary sheath of nerves possesses such a structure has been demonstrated by Hardesty.² This fact explains why the axis-cylinders of medullated nerve fibers, despite the fact that they are enveloped, even

¹ J. Loeb: *Arch. f. d. ges. Physiol.*, lxxxviii, p. 68, 1901; cvii, p. 252, 1905; *Amer. Journ. of Physiol.*, iii, p. 327, 1900; vi, p. 411, 1902.

² Irving Hardesty: *Amer. Journ. of Anat.*, iv, p. 329, 1905. These investigations of Hardesty's dispose at once of one of the chief assumptions involved in Overton's and Meyer's hypothesis of narcotic action; so long as we supposed the conducting elements of the nervous system to be for the most part buried in a continuous layer of lipid, no poison could possibly reach them which was not soluble in lipid. Hardesty having demonstrated the existence of a network of protoplasmic fibrils in this fatty sheath, however, it is evident that even substances insoluble in lipid could reach all parts of the nervous system, the network of fibrils acting as the solvent and conducting medium.

at the nodes of Ranvier, by lipid material,¹ nevertheless stain deeply in methylene blue, which is insoluble in lipoids, and leaves the medullary sheath itself unstained. The outer layer of the medullary sheath is a fibro-lamellar membrane, fibrils from which intersect and form a network throughout the lipid material. These fibers conduct the stain to the axis-cylinder and may even under certain circumstances be seen to be faintly stained themselves.²

Such a structure as the one which I have outlined, a homogeneous protein surface membrane with a discontinuous fatty layer underneath, would appear to be very general. It may be seen clearly in the sea-urchin egg when, in the process of artificial fertilization, the egg is treated with fat-solvents.³ The fatty layer next the surface takes up the fat-solvent, at the same time, probably, absorbing water,⁴ and the superficial hyaline protein membrane is thereby lifted up from the surface of the cell and rendered visible. It may be here pointed out that it is not the most superficial layer of the egg-cell which is dissolved by the fat-solvent in artificial parthenogenesis but fatty constituents which lie beneath the superficial layer.

Similar phenomena are very frequently seen when infusoria such as *Paramœcia* are cytolyzed by certain poisons (benzene, toluenè, xylene; chloroform, ether, etc., as well as colchicin, quinin, nicotin and other substances); clear vesicles are formed at the surface of the organism, these are surrounded by a clear, homogeneous, fairly tough membrane which has been lifted up from the surface of the cell by the fluid collected underneath.

¹ Hardesty: *loc. cit.*

² Hardesty: *loc. cit.*

³ J. Loeb: *Arch. f. d. ges. Physiol.*, ciii, p. 257, 1904; *Univ. of Calif. Publ.*, Physiol, ii, p. 113, 1905; *ibid.* iii, p. 61, 1907; Address delivered at the Seventh International Zoölogical Congress, Boston, August 22, 1907.

⁴ It may here be noted that if the fat were present in this case in the form of approximately spherical droplets packed as closely as possible, increase in the size of these droplets through the taking up of the lipid-solvent would result in an increase in the size and absolute volume of the interstices between them (Barlow: *Nature*, xxix, p. 186, 1883; Kelvin: *Proc. Roy. Soc. Edin.*, xvi, p. 693, 1889; Osborne Reynolds: *Phil. Mag.*, 1885; *Scientific Papers*, Cambridge, ii, p. 203; *Proc. Roy. Institution of Great Britain*, 1886; *Scientific Papers*, ii, p. 217), and water would necessarily flow in to fill up these interstices.

IV. CONCLUSIONS.

(1) Experiments are described upon the influence of acid and alkali on the relative solubilities of dyes in water and in lipoids. The lipoids used were ethyl acetate, butyrate and benzoate; triacetin and, in certain experiments, triolein and olive oil. It was found that the solubility of an acid dye in lipoid is increased by the addition of acid while that of a basic dye is increased by the addition of alkali. The interpretation which is placed upon these results is that the free color-acids or color-bases are more soluble in lipoid than their salts. No marked difference exists between the acid and basic dyes in this respect, the free color-acids being about as soluble in lipoid as the free color-bases. The *salts* of the basic dyes, however, are, as a rule, soluble to some extent in lipoid, while those of the acid dyes, as a rule, are not.

(2) Applications of the above results to the use of indicators are suggested.

(3) When fat-cells in the fresh omentum stain at all they behave towards the dye exactly as does the ethyl acetate in the experiments described above. There is no correspondence between the depth of stain of connective-tissue elements and the fat-cells.

(4) Red blood corpuscles, fixed by a method described in the paper, when they stain at all, behave towards the dye somewhat as does ethyl acetate, but, as a rule, they only stain in acid dyes and their power to stain in a dye cannot be predicted from their lipoid solubility.

(5) The addition of proteins (casein, gelatin, protamin) to watery solutions of dyes greatly alters the distribution of the dye between water and lipoid and this alteration is characteristic for each protein. Hence proteins combine chemically with certain dyes.

(6) It is pointed out that Overton's theory of a lipoid limiting membrane at the surface of cells in conjunction with a "solid solution" theory of staining is untenable.

(7) It is pointed out that Overton's theory that a lipoid membrane envelopes the surface of living cells is founded upon three classes of evidence, namely:

- (i) The phenomenon of "rounding up" of droplets of protoplasm when squeezed out of cells into a watery liquid.
- (ii) The relation which is stated to exist between the lipid-solubility of a narcotic, poison, or dye, etc., and its effect upon living matter.
- (iii) The relation which is stated to exist between the osmotic pressure of a solution and its toxic or plasmolytic action, demanding the existence at the surface of the cell of a semipermeable membrane.

(8) It is shown that the assumption of an oily layer at the surface of cells or of protoplasmic droplets is quite gratuitous in so far as the maintenance of its integrity is concerned, and that the properties of concentration-films of protein quite suffice to account for the phenomena observed. It is moreover shown that such films exist very generally, probably universally, at the bounding surface of droplets of protein solution.

(9) It is shown that the supposed relation between the lipid solubility of a narcotic, poison, dye or salt and its effect upon living matter is one which will not bear a critical examination, which certainly does not hold good for dyes and which probably, therefore, does not hold good for the majority of substances which may exert an influence upon living matter.

(10) It is pointed out that previous observers have demonstrated that the influence of the concentration of a medium upon the cells or organisms suspended in it cannot be attributed to purely osmotic factors and that, on the contrary, osmotic pressure plays a very subordinate rôle in phenomena of toxicity.

(11) It is suggested, and reasons are brought forward for supposing that at the surface of every living cell there exists a thin, optically homogeneous, sparingly soluble, protein membrane, and that in many cases a discontinuous lipid layer may underlie this.

(Postscript added at the time of proofreading, December 4, 1907.)

Since writing the above I have read a paper by Loomis¹ in which he describes extensive experiments upon the influence of

¹ H. M. Loomis: United States Department of Agriculture, Bureau of Chemistry. Circular No. 35, 1907.

acid and alkali upon the partition of dyes between water and various other solvents. Among other solvents he used ethyl acetate. Most of the dyes which he used are dyes of technical importance and are not those usually used in histological and cytological studies. Such of his experiments, however, as were made upon the same dyes as mine are identical in their results with those described above.

I have also investigated the solubility of certain dyes in a benzene solution of Kahlbaum's lecithin.¹ The solution used was a nearly saturated one made by shaking up lecithin in benzene for a considerable period. An aqueous neutral solution of methylene blue, shaken up with this solution, communicates to it a faint blue color, which it does not to benzene alone. Particles of suspended lecithin are also stained a faint blue. Methyl green imparts to the benzene-lecithin solution a deep red-violet color, whereas it does not impart any color at all to benzene alone. If, however, the methyl green be previously extracted with chloroform to remove all the methyl violet it no longer imparts any color at all to the benzene-lecithin solution whether in acid, neutral, or alkaline solution. As I have pointed out above, it is the methyl green and not the methyl violet which chiefly stains the living cell. Congo-red, and sulphindigotate of sodium, whether in neutral, acid, or alkaline solution are insoluble in the benzene-lecithin solution.

¹ Similar to that used by Overton in his investigation. *Jahrbücher für wiss. Botanik*, xxxiv, p. 669, 1900.

NOTE ON "ADSORPTION" AND THE BEHAVIOR OF CASEIN IN ACID SOLUTIONS.

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California.)

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In a recent paper¹ van Slyke and van Slyke have described results of considerable interest and importance upon the extraction of acid from watery solutions by means of casein. In the theoretical consideration of their results, however, they have decided, it seems to me, upon somewhat insufficient evidence, that the taking up of acid from watery solution by casein is a process of "adsorption."

If "adsorption" be defined as the expression of a "mechanical affinity," in other words of an atomic attraction which is different in degree if not in kind from that which we call chemical affinity, we cannot venture to assert that any given phenomenon is an "adsorption phenomenon" until we have definitely excluded the possibility of its being a purely physical phenomenon (such as concentration at the surfaces of two phases,² "solid solution," etc.) or a purely chemical phenomenon or simply a mixture of purely physical and purely chemical phenomena (such as certain reactions in heterogeneous systems, where the rate of diffusion across the interface of two phases determines the velocity of the chemical reaction³); or until we

¹ van Slyke and van Slyke: *Amer. Chem. Journ.*, xxxviii, p. 393, 1907.

² J. Willard Gibbs: "Equilibrium of Heterogeneous Substances," *Trans. Connecticut Acad. of Sciences*, 1878, reprinted in *The Scientific Papers of J. Willard Gibbs*, i, p. 219, etc., 1906; Rayleigh: *Scientific Papers*, iii, p. 351, 1902; F. G. Donnan: *Zeitschr. f. physikal. Chem.*, xxxi, p. 42, 1899. For interesting examples of such surface concentration, see W. P. Dreaper: *Journ. Soc. Dyers Colorists*, xxiii, p. 188, quoted after *Chemical Abstracts*, p. 2302, Sept., 1907.

³ Nernst: *Zeitschr. f. physikal. Chem.*, xlvii, p. 52, 1903; Brunner: *Ibid.*, xlvii, p. 56, 1903.

ter of casein it is evident that for a given mass of casein we cannot consider the concentration of the CASEIN (OH)_y in the above equation as constant, inasmuch as only a certain proportion of the casein is acting as a base and, as I have shown in the case of serum globulin,¹ this proportion varies with the acidity of the solution; the greater the concentration of hydrogen ions in the solution the greater is the acid-binding power of the protein.

If we consider, moreover, that casein when shaken up in dilute acid solutions forms a two-phase system (since, except in the case of acetic acid, a small amount of the casein always dissolves in the external solution) it is possible that the two phases represent relative minima of dissociation of the casein and of its polymeric modifications, in which case we should have for the hydrogen ion concentrations in the two phases:

$$\frac{a_1}{a_2} = \sqrt{\frac{k'_a k''_b}{k''_a k'_b}}$$

where a_1 and a_2 are the respective hydrogen ion concentrations and k'_a, k'_b , etc., are proportional to the acid and basic dissociation constants of the ampholytes which are at minimum of dissociation in each phase, while in the case of casein suspended in sulphuric acid the problem is complicated owing to the fact that the possible number of minima of dissociation is greatly increased owing to the presence of the divalent ion.²

The fact, therefore, that the theoretical relation deduced by van Slyke and van Slyke does not hold good cannot, in itself, be held as conclusively indicating that the binding of acid by casein is not a chemical phenomenon since the data from which they derive the relation are not certain; on the contrary they are in direct contradiction to certain characteristic properties of proteins.

Coming, therefore, to the criteria of "adsorption" which are advanced by van Slyke and van Slyke, following van Bemmelen,³

¹ T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, p. 437, 1907.

² T. Brailsford Robertson: *Journ. of Physical Chem.*, x, p. 524, 1906.

³ van Bemmelen: *Zeitschr. f. anorg. Chem.*, xxxvi, p. 381, 1903; *Zeitschr. f. physikal. Chem.*, xviii, p. 33, 1893.

Ostwald¹ and others, we search in vain for one which cannot be displayed by one and the same chemical or physical and chemical phenomenon. They are as follows:

(1) "The adsorbed amounts stand in no equivalent proportions to the adsorbing mass."

This is a characteristic of incomplete reactions; not unless reactions are complete do stoichiometric relations between the reacting substances appear other than indirectly in our quantitative results. More than ever is this the case if, in addition to the reaction being incomplete, the active mass of one of the components varies with the active mass of the other; as is the case when amphoteric electrolytes are treated with acids or alkalies.²

(2) "The composition varies with the structure of the adsorbing substance and with all the modifications which the latter undergoes by reason of its method of preparation, age, heat, or the action of any other substance." This is a phenomenon not unfamiliar in chemical reactions between unstable chemical entities which are liable to undergo secondary reactions upon keeping or upon treatment by heat, etc. Moreover, this factor did not enter into van Slyke and van Slyke's experiments, as they used uniformly prepared material of uniform age.

(3) "The composition varies with the temperature."

It is a fact not unfamiliar to chemists that heat very frequently shifts chemical equilibria.

(4) "The composition varies with the concentration of the solution, in case substances are adsorbed from their solutions; and the composition varies in such a manner that the adsorption factor k in the formula $\frac{c_1}{c_2} = F(k)$ is not constant but is dependent upon the concentration of the solution according to a function that we do not understand. In general, however, c_1 increases more slowly than c_2 , where c_1 and c_2 are the amount of solute adsorbed per unit mass of adsorbent, and the concentration of solute in solution respectively, and F is an unknown function." van Bemmelen adds, however, that "when the adsorp-

¹ Ostwald: *Lehrbuch d. allgem. Chem.*, 2 Aufl., i, p. 1096, 1903.

² T. Brailsford Robertson: *Journ. of Physical Chem.*, x, p. 524, 1906; xi, p. 437, 1907.

tive power is small and the solution is not concentrated then, between certain limits of concentration $\frac{c_1}{c_2}$ is nearly constant."

Most of the exponents of the adsorption hypothesis, however, agree in stating that the relation between c_1 and c_2 can be expressed by the equation $\frac{c_1^p}{c_2} = \beta$, β and p being constants which are peculiar to the system.¹ As I will show in the sequel this relation could be found in purely physical and chemical phenomena where no suspicion of "adsorption" can exist. But it is evident

that in heterogeneous systems the simple relation, $\frac{c_1}{c_2} = \text{const.}$, must necessarily be unusual or restricted in its applicability. In no sense can the above very general statement of the relation between c_1 and c_2 be held to represent a criterion of adsorption to the exclusion of purely physical and chemical factors.

(5) "The velocity with which the formation of an adsorption compound occurs decreases continually as more substance is adsorbed and the adsorption approaches equilibrium."

This is a characteristic which applies equally well to all reversible chemical reactions and to all phenomena the velocity of which depend upon the *number* of molecules of a given species which are present in the system and which involve the disappearance or the carrying out of the sphere of action of molecules of that species.

The "criteria" so far quoted are those given by van Bemmelen and quoted by van Slyke and van Slyke; in addition to these van Slyke and van Slyke adduce two criteria due to Ostwald.

(6) "The reaction approaches a true equilibrium which can be approached from either side."

This is also true of all reversible chemical reactions.

(7) "Using rectangular coördinates, if one plots dilution of the solution as ordinates and amounts of solute adsorbed as abscissæ, the resulting curve will be nearly a hyperbola."

¹ Schmidt: *Zeitschr. f. physikal. Chem.*, xv, p. 56, 1894; Walker and Appleyard: *Journ. Chem. Soc.*, xcix, p. 1334, 1896; Freundlich: *Zeitschr. f. physikal. Chem.*, lvii, p. 385, 1906; *Zeitschr. f. Chemie u. Industrie der Colloide*, i, p. 321, 1907.

This is merely a graphical restatement of criterion number 4.

To the above "criteria" we may add the fact that most of the so-called "adsorption" combinations hitherto described have a low temperature-coefficient. Arrhenius and van't Hoff have shown that most chemical reactions have a temperature-coefficient of two or more, that is, the velocity of the reaction is doubled or more than doubled by a rise of 10° in the temperature of the system. No physical phenomenon has its velocity increased by nearly this amount by a similar rise in temperature and when physical phenomena determine the velocity of a chemical reaction, for example when the progress of the reaction in a two-phase system depends upon the diffusion across the interface of the phases of one of the reacting substances, then the temperature-coefficient is low, sometimes nearly approximating to that of a purely physical phenomenon, but the degree to which it will do so cannot be predicted but must be determined separately for each reaction.¹ Bayliss found for what he considered a typical case of adsorption a temperature-coefficient of 1.36.² The results of van Slyke and van Slyke given in their Tables XII and XIII indicate a low temperature-coefficient for the process of the taking up of acid from solution by casein.

In further support of their hypothesis van Slyke and van Slyke have also alluded to the fact that the acid bound by casein is readily extracted again by water, but this argument, as I have elsewhere pointed out,³ proves nothing, inasmuch as the sulphuric or hydrochloric acids can be readily washed out by water from such undoubtedly chemical compounds as mercuric sulphate and cupric chloride.⁴ Hence we see that none of the criteria advanced by the exponents of adsorption can, singly, be taken as proving that a given phenomenon is an adsorption phenomenon and not purely physical, chemical, or physical and chemical in character. If, however, it be argued that it is not one of the above criteria but their sum which differentiates an adsorption phenomenon from a purely physical or a purely chemical or a

¹ Senter: *Journ. of Physical Chem.*, ix, p. 311, 1905.

² Bayliss: *Biochem. Journ.*, i, p. 175, 1906.

³ T. Brailsford Robertson: this *Journal*, ii, p. 378, 1907.

⁴ Lescoeur: *Ann. de chim. et de phys.*, 7 ser, ii, p. 97, 1894; Haywood: *Journ. of Physical Chem.*, i, p. 411, 1897.

physical and chemical phenomenon we may cite one of the simplest possible combinations of physical and chemical phenomena to which *all* of the above criteria apply.

Consider a substance A which distributes itself between two immiscible solvents X and Y and which forms with Y a compound AY which is soluble in Y but insoluble in X; it is required to find the proportion of A which, for different concentrations of A, will be found in the solvent Y. In the first place, for simplicity, we will assume that the presence of the compound AY in the solvent Y does not alter the solubility of A in Y. Let a_1 and a_2 be, respectively, the total amounts, combined and uncombined, of A in Y and in X and let z be the concentration of the compound AY in the solvent Y and b the concentration of *uncombined* A in Y. Then we have, by the mass-law, if the amount of the compound AY is small, so that the active mass of Y may be considered as constant; $z = kb$, where k is a constant and $a_2 = k' b$ where k' is the partition-coefficient of A between X and Y. Hence since $a_1 = b + z$ we have:

$$a_1 = b(1+k) \text{ and } \frac{a_1}{a_2} = \frac{1+k}{k'}$$

It is obvious that van Bemmelen's first criterion applies to this system; the amount a_1 of the substance A which enters the solvent Y will stand in no equivalent proportions to the mass of Y because, the reaction $A + Y \rightleftharpoons AY$ being a balanced one, neither A nor Y is ever completely bound, and, besides, A enters Y in excess of the amount which is bound and this excess varies with the total concentration of A in the system and cannot therefore stand in definite stoichiometrical relation to Y. A stoichiometrical relation between A and Y in the compound AY nevertheless exists, but is concealed in our quantitative results.

That van Bemmelen's second criterion should apply it is only necessary to suppose, in addition, that the substance Y is an unstable one which is very liable to undergo modifications in its solvent-power owing to secondary chemical reactions.

That van Bemmelen's third criterion would apply is evident, for the value of k and, not improbably, of k' would vary with temperature and, consequently, $\frac{a_1}{a_2}$ would vary with temperature also.

We have seen that $\frac{a_1}{a_2}$ in the simple case which we have considered is constant, a particular case of the general formula $\frac{c_1^p}{c_2} = \beta$ found by Freundlich¹ and others for adsorption phenomena; were *two* molecules of A to combine with Y and the amount combined with Y were large compared with the amount simply dissolved in Y then we should obtain the approximate formula $\frac{a_1^2}{a_2} = \text{const.}$ If, even in the simple case we have considered, we take into consideration the fact that the presence of the compound AY will probably diminish the solubility of A in Y, since the addition of other bodies usually diminishes the solubility of any substance in a solvent,² then k' will increase as a_1 increases and hence a_1 will diminish as the concentration of A in the solvent X increases. If, also, the compound AY is slightly soluble in X and if X and Y are slightly miscible in each other, and if we take into consideration the fact that the active mass of Y is progressively diminished by the formation of the compound AY, obviously all the possible relations comprehended in van Bemmelen's criterion 4 could be realized.

Criterion 5 will also, obviously, apply to the case under consideration, since the reaction $A + Y \rightleftharpoons AY$ will proceed more and more slowly as equilibrium is approached, and the velocity with which A is taken up by Y will depend both on the velocity with which A diffuses in the two solvents and upon the velocity of the chemical reaction.

Criterion 6 also, obviously, applies.

Criterion 7 is, as I have said, merely a restatement of criterion

4. Since, in the simple case considered above $\frac{a_1}{a_2} = \text{const.}$, therefore if v be the dilution of A in X then $a_1 \times v = \text{const.}$ and if v be plotted vertically and a_1 horizontally we shall obtain a hyperbola. The other possibilities presented by the system, due to the solubility of AY in X, etc., would, if similarly plotted, give

¹ Freundlich: *loc. cit.*

² For instances, see Gustav Mann, *Physiological Histology*, Oxford, p. 329, 1902.

similar curves. The temperature-coefficient of the reaction would depend upon the degree of fineness with which Y is dispersed through X. If X and Y were separated by a small surface of contact the temperature-coefficient would be low, that of diffusion; if they were separated by a very large surface, that is, if Y were distributed through X in a state of division of almost molecular fineness, the temperature-coefficient would be that of a chemical reaction. Intermediate degrees of dispersion would give intermediate temperature-coefficients.

Finally, the substance A could be completely extracted from the solvent Y by repeated extractions with the solvent X.

Thus we see that each and every one of the criteria proposed by the advocates of "adsorption" for the phenomena which they classify under this head apply equally well to the very simple system which I have outlined above, a system which may be approximately illustrated by the partition of chlorine between carbon tetra-chloride and water.¹

If, examining the evidence hitherto brought forward in defense of "adsorption," we inquire what is the real mental difficulty which these investigators have encountered in the way of a chemical explanation of the phenomena, we find that in many cases it is not improbably simply a difficulty of conceiving a chemical combination between the bodies under investigation.² Nevertheless it may be suggested that if chemists had from the beginning proceeded with this mental bias we should have discovered but few chemical compounds, and it may also be pointed out that at surfaces and, consequently, in two-phase systems reactions may take place which do not take place in homogeneous systems.³ Moreover this difficulty does not apply to a purely physical conception of the phenomena as examples of surface-concentration at the interface of two phases due to the reduction thereby of the potential energy stored up in the surface.⁴ It

¹ A. A. Jakowkin: *Ber. d. deutsch. chem. Gesellschaft.*, xxx, p. 518.

² van Slyke and van Slyke, however, started their investigation under the impression that the union between acid and casein was due to the formation of a definite chemical compound.

³ Liebreich: *Zeitschr. f. physikal. Chem.*, v, p. 529, 1890; vii, p. 83, 1891.

⁴ This is the view of "adsorption" phenomena which has been very clearly expressed by Freundlich *Kapillarchemie und Physiologie.* Dres-

appears not improbable that in chemical phenomena we have to deal with atomic, while in physical phenomena we have usually to deal with molecular attractions or with the attractions of matter in mass; on the other hand, recent discoveries seem to point to the fact that the discontinuities of the weight-function for atoms and, therefore, the stoichiometrical relations between them are phenomena which depend upon the fundamental structure of matter:¹ we should therefore expect, *a priori*, to find an emphatic discontinuity between physical and chemical phenomena and any alleged examples of transition phenomena must be subjected to rigid scrutiny and only accepted as such after all the possibilities offered by purely physical or chemical phenomena or combinations of these have been tested and found insufficient to account for the phenomena. The procedure of the advocates of "adsorption" has been the reverse of this; with hardly an endeavor to critically examine the other possibilities of the systems with which they deal they group together a mass of doubtless wholly unrelated phenomena as "adsorption" phenomena and instances of "mechanical affinity" simply because they do not present the quantitative and qualitative properties of the very simplest and most obvious chemical or physical phenomena.

Therefore, in the absence of any specific criteria and in the absence of any exhaustive exclusion of other and not less probable physical and chemical possibilities, it may be questioned whether "adsorption" (or "mechanical affinity") has yet been proved to exist at all. However this may be, it certainly appears premature at the threshold of our physico-chemical knowledge of proteins to declare a group of their compounds or reactions to be "adsorption-compounds" or "adsorption reactions" and to thereby, and without further discrimination, group them with phenomena which are, not improbably, essentially different in character and in mechanism, and to import into a field already sufficiently obscure a conception so misty as that of "mechanical affinity."

den, 1907. It is quite possible that the taking up of acid from dilute solutions by suspended casein is a phenomenon of this purely physical nature.

¹ J. J. Thomson: *Phil. Mag.*, series 6, vii, p. 237, 1904.

FURTHER STUDIES ON PUTREFACTION.¹

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In the present paper the writer wishes particularly to emphasize a number of points brought out in a previous publication,² and to record certain observations which have recently been made regarding the presence of putrefactive anaërobes in the human intestine.

Although Bienstock's contention³ that obligate anaërobes alone can bring about putrefactive changes in native proteids met with much opposition, his observations are today pretty well substantiated. It is true that here and there an investigator still claims that he has demonstrated this property in certain aërobes or facultative anaërobes, but there is lacking the evidence, either that the transformation was strictly one of putrefaction, or that the organism in question was not mixed with some contaminating anaërobe.

Fischer⁴ obtained an organism from a case of malignant stomatitis (*Bacterium stomato-fætidum*) which according to him is a putrefactive organism, and yet an aërobe. This bacterium resembles the diphtheria bacillus in morphology; it is motile, Gram-negative, and does not form spores. It grows well on all ordinary media and causes abundant gas production in dextrose and saccharose, but not in lactose. Although Fischer calls it an aërobe, he states that *some* growth takes place in an atmosphere of hydrogen or carbon dioxide. A number of different proteids were decomposed with the formation of putrefactive products.

¹ The work on which this paper is based was aided by an appropriation from the Rockefeller Institute for Medical Research.

² Rettger: This *Journal*, ii, p. 71, 1906.

³ Bienstock: *Arch. j. Hyg.*, xxxvi, p. 335, 1899; *ibid.*, xxxix, p. 390, 1901.

⁴ Fischer: *Zeitschr. j. Hyg.*, xlix, p. 329.

The statement is made that examinations by the vacuum method for contaminating anaërobes proved negative.

Should the above observations be founded on actual facts, then exceptions must be made to the proposed rule that anaërobes alone can cause putrefaction. The possibility, however, of the above organism being mixed with one or another of the so-called "ubiquitous" anaërobes can not help but impress itself on the reader.

I have within the last two years repeatedly examined at least sixty aërobes and facultative aërobes or anaërobes for putrefactive products, and in every instance obtained strictly negative results. The tests were made under different conditions of temperature, reaction, etc. When grown in unquestionably pure culture, there was no reduction in the bulk of the proteids employed, nor were there any other signs of decomposition. When mixed with one or more of the putrefactive anaërobes, the mooted transformation occurred, even when the cultural conditions did not approach those of complete anaërobiosis.

No one can deny that much of the decomposition of albuminous matter in nature is carried on by the obligate aërobes (*B. subtilis*, *B. mycoides*, etc.), but such transformation is one of ordinary dissolution or digestion. Free oxygen is always needed in abundance. This can easily be seen in ordinary test tube experiments. If we place cubes of coagulated albumin (egg or serum) in the depths of tubes containing bouillon or some other not unfavorable liquid medium, very little or no dissolution of the proteid will be observed until enough of the liquid has been removed by evaporation to expose the surface of the albumin to direct contact with the air.

Decomposition of albumin by the aërobes is never accompanied by the foul odors which are so characteristic of putrefaction. By "putrefaction" is meant here, as elsewhere in the paper, what is called "Fäulniss" in the German language.

I have never tested the group of thermophile bacteria as to their ability to cause putrefaction, and whether or not Bienstock's rule that putrefaction is limited to obligate anaërobes applies here must be further investigated. Quite recently Bardou¹ made a study of the thermophile bacteria which he

¹ Bardou: Thesis, Ref. *Centrabl. f. Bakt. Parasitenk.*, etc., i, Referate, xxxix, p. 744, 1907.

regularly found in the septic tank of the sewage purification works at Lille. Among those isolated, four appeared to be of much importance in that they had a strong digestive action on certain native proteids (egg and serum albumin, fibrin, and vegetable albumin) when kept under anaërobic conditions. It is not stated in the abstract whether this decomposition is one of putrefaction or merely of the ordinary digestive or tryptic sort; neither is it stated whether the organisms are obligate or facultative anaërobes. Reference to the original paper only will determine these points.

In summing up the more important data on this subject, one conclusion is paramount, namely; real putrefaction is the work of obligate anaërobes. If certain exceptions do occur, which has not been demonstrated with any degree of certainty, they are indeed very few, and may be safely disregarded in a general consideration of this subject.

Not all obligate anaërobes have this property of producing putrefaction. The strict anaërobes which have come under my observation may be divided into four classes, in so far as their biochemical characters are concerned. First, those that produce very little or no putrefactive change or fermentation with evolution of gas. Perhaps the best example of this class is the tetanus bacillus. Second, those that have a strong putrefactive action on native proteids but fail in fermentative properties, as illustrated by *B. putrificus* of Bienstock. Third, those which are primarily fermentative organisms, and whose putrefactive functions¹ are very slight or perhaps absent; example, *B. aërogenes capsulatus* of Welch or *B. enteritidis sporogenes* of Klein. And fourth, those which have very marked putrefactive and fermentative properties, as shown best in the bacillus of malignant edema and the bacillus of symptomatic anthrax.

It seems strange that the tetanus bacillus which is so commonly associated in nature with decaying organic matter should not possess the ability to attack native proteids, at least in a significant manner. Numerous strains of this organism have been tested by me within the past two years, relative to this point,

¹ By these terms I mean again the ability to decompose native proteids with the formation or liberation of foul-smelling products—mercaptan, aromatic oxy-acids, etc.

but in not a single instance was there any apparent action on the egg-meat mixture or plain coagulated egg albumin. It might be argued that the organisms examined may have possessed this function at one time, but that they had since lost it in consequence of the newer conditions to which they had been subjected. This may be true in a number of instances, but most of the strains tested were those that were isolated quite recently from soil, barnyard dirt, etc., and in every way appeared to be true tetanus bacilli.

Bacillus putrificus has a very vigorous action on native proteids. The intensity of this action may vary, however, depending on the age of the strain and the conditions under which it is kept for any length of time. The original culture which I isolated from street dirt gradually lost its putrefying properties when grown on the ordinary laboratory media, so that in a year's time it was unable to make any marked impression on the egg-meat mixture or plain coagulated egg albumin. Eventually it refused to grow in any of the common media, and I was unable to resuscitate it. Dr. Herter informed me that he had had somewhat similar experience with the putrificus bacillus. On the other hand, all the strains of the bacillus of symptomatic anthrax and the bacillus of malignant edema seemed to retain their putrefactive property, and the organisms appeared to be more hardy and long-lived.

In an extended investigation as to the comparative properties of *B. œdematis maligni* and *B. anthracis symptomatici*, I have been unable to find any constant differential characters aside from their respective pathogenicity. This is particularly true regarding the nature and rate of putrefaction and fermentation, and the products formed. The morphology and biochemical characters of these organisms as well as of the putrificus bacillus are quite variable, as Achalme¹ and others have shown, and in order to obtain analogous results they must be grown in the same media and under the same conditions. In the egg-meat mixture which I have been employing regularly, the morphology and biochemical properties are quite constant. Here the putrificus organism always assumes the long slender bacillus or drumstick form, the spores being quite large, almost perfectly round and

¹ Achalme: *Ann. de l'Inst. Pasteur*, xvi, p. 633, 1902.

situated at the extremity of the bacillus. In the same medium the rods of the malignant edema and the symptomatic anthrax bacillus are much shorter and thicker, and are located not at, but towards, the end of the bacilli, giving the latter an almost spindle-shaped appearance. When free the spores are seen to be much smaller and decidedly less round than those of *B. putrificus*.

An agar culture of an anaërobe which was sent to the laboratory, labelled *B. botulinus*, Kral, appeared in all of the above-mentioned characters to be identical with the malignant edema and the symptomatic anthrax bacillus. Its putrefactive and fermentative properties were as marked as those of the two other organisms; and as to its morphology, it would certainly have been difficult to distinguish it from them. Whether this organism was the one which was originally isolated as *B. botulinus*, and not a contamination form, it is impossible to state.

The question as to whether the *Bacillus aërogenes capsulatus* of Welch has any pronounced putrefactive action on proteids is an important one. I have examined fully ten strains of this obligate anaërobe which were either obtained from some of the prominent laboratories of this country or directly isolated from feces. Their action was tested on egg-meat mixture, coagulated egg and serum albumin and casein. While there appeared to be a slight decrease in the bulk of the proteids in some instances, as well as the production of quite disagreeable odors, the transformation never assumed the character of real putrefaction. The unpleasant odor which was observed was not like that of mercaptan, etc., but was probably due to the presence of butyric and closely-allied acids. Such decomposition of the medium may take place in the absence of carbohydrates, the proteids themselves undergoing some change, as has been shown by other investigators.

Tissier and Martelly¹ isolated an anërobe from putrefying meat which resembled the Welch bacillus, *B. perfringens*, and which they claimed had a decided putrefying action on blood fibrin. From their paper we are led to believe, however, that the action is not a very rapid one. Herter² also ascribes marked putre-

¹ Tissier and Martelly: *Ann. de l'Inst. Pasteur*, xvi, p. 865, 1902.

² Herter: *Bacterial Infections of the Intestinal Tract*, 1907.

factive properties to the bacillus of Welch and regards it as being largely responsible for certain intestinal disturbances. He uses the term putrefaction in a much broader sense, however, than I do.

Certain investigators (Nencki, Zoya, Kerry, Bienstock) have observed that indol and skatol are absent from the products of the putrefying anaërobes. Passini makes an exception to this rule and asserts that the bacillus of gaseous phlegmon produces an abundance of indol when grown in pure culture on blood serum. I have been unable to find any indol whatever by the usual nitric acid test among the products of *B. putrificus*, *B. œdematis maligni* and *B. anthracis symptomatici*. By the use of Herter's β -naphthaquinone-sodium-monosulphonate test, however, minute traces of indol were detected on different occasions. It appears quite certain that the production of indol is not an important function of the putrefying organisms or anaërobes in general.

PUTREFACTION IN THE HUMAN INTESTINE.

Since Bienstock's assertion¹ that *B. putrificus* is never present in the feces of normal individuals, the subject of intestinal putrefaction has been investigated with renewed effort.

Salus² states that he was able to find spores of putrefying anaërobes, but only in small numbers. He therefore believes that while the organisms may exist in the intestine, they are there in spore form and do not undergo multiplication until after they leave the body.

Tissier and Martelly³ claim to have found and isolated an anaërobe similar to Bienstock's putrificus bacillus from meconium. Passini⁴ also seems to have detected the same organisms in meconium, on certain occasions. In the stools of breast-fed infants *B. putrificus* could be found only when several loopfuls of material were taken for examination. Very few spores were present. In bottle-fed children this anaërobe was slightly more

¹ Bienstock: *Arch. f. Hyg.*, xxxix, pp. 390-427, 1901.

² Salus: *Arch. f. Hyg.*, li, p. 97, 1904.

³ Tissier and Martelly: *Ann. de l'Inst. Pasteur*, xvi, p. 865, 1902.

⁴ Passini: *Zeitschr. f. Hyg.*, xlix, p. 135, 1905.

common than in the breast-fed, and in the normal adult the organism occurred in abundance, particularly in the form of spores.

According to Mace¹ the bacillus of malignant edema is a constant inhabitant of the intestinal tract. Passini was unable to substantiate this.

In a more recent publication Bienstock² announces that he was again unable to find any spores of *B. putrificus* in the feces of normal persons, as well as a large number of hospital patients. By the use of a special medium, however, he found an organism resembling his putrificus bacillus in many respects, but differing from it in that it readily attacks sugars with the formation of acetic, butyric, lactic and carbonic acids. It has marked putrefactive and fermentative properties, and was found by Bienstock in 20 per cent of the stools examined. He gave it the name of *B. para-putrificus*.

In my previous paper on putrefaction I mentioned the fact that on two or three occasions organisms were seen in egg-meat cultures made from human feces which in their biochemical aspects resembled *B. putrificus* and the bacillus of malignant edema. Since these organisms are so abundant in nature (dust, dirt, soil, etc.) and since they were seen in only a few samples of the feces examined, it was concluded that they were either present incidentally or were mere contamination forms.

Since then I have examined at least fifty specimens of what might be called normal feces. An effort was made to determine the extent to which native proteids were decomposed, and in the second place to detect one or more of the well-known putrefactive anaërobes which were responsible for this decomposition. To this end the following method was employed.

Known quantities of fresh feces were introduced into large test tubes containing 10 to 12 cc. of the egg-meat mixture previously described.³ After distributing the fecal matter as thoroughly as possible by means of a stout platinum wire, the tubes were heated at 80° C. for 10 minutes, rendered anaërobic by the Wright method and incubated at 37° C. The tubes were

¹ Quoted from Passini, *loc. cit.*

² Bienstock: *Ann. de l'Inst. Pasteur*, xx, p. 497, 1906.

³ Rettger: *This Journal*, ii, p. 71, 1906.

daily examined for signs of putrefaction, but they were not opened until the sixth or seventh day. Experience soon showed that in this medium the putrefying anaërobes could usually be detected between the fourth and seventh day, by their characteristic spore forms. At other times it may be difficult to detect them.

The amount of fecal matter used was roughly estimated by means of three "standard" platinum loops which were made and reserved for that purpose. The smallest loop (*a*) held approximately 2 milligrams of feces; the second (*b*) about 8 milligrams, and the largest (*c*) 32 milligrams. In this way there was less chance of contamination than if weighed quantities of material had been taken and suspended in water or salt solution. The results of some 54 examinations are summed up in the following table:

TABLE SHOWING THE REDUCTION OF SOLID MATTER IN PER CENT, AND THE PRESENCE OF *B. PUTRIFICUS* AND ORGANISMS OF THE MALIGNANT EDEMA BACILLUS TYPE.

Feces	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
<i>a</i> (2mg.)	20	40	12	30	0	0	0	0	0	0	0	0	8	0	10	0	10	5
<i>b</i> (8 mg.)	15	40	50†	40	35†	15	0	0	0	0	60*	30	7	60*	30	8†	10	10
<i>c</i> (32 mg)	10	60*	15	75†	0	35†	9	6†	40†	0?		50*	15	25†	35†	10†	15†	10

†Indicates the presence of *B. putrificus*.

*Indicates the presence of *B. edematis maligni*.

A glance at the table will show that the putrificus bacillus was found in 11 samples, or 20 per cent of all the tubes examined; and bacilli of the malignant edema type in 4 specimens, or 7.4 per cent of the tubes examined. It is quite probable that these figures are too low, as the organisms in question may have escaped detection in some of the tubes, particularly where the reduction in the amount of insoluble proteid was so large.

It will be seen, too, that there is a marked difference in the three series, *a*, *b* and *c*, and that the results are influenced by the amounts of feces used in the tests. Not one of the 18 tubes in series *a* was found to contain the putrefying organisms mentioned, while two-thirds of the positive results were obtained in the tubes which had received 32 milligrams of feces.

Quite a number of examinations were also made of unheated specimens of feces. It was soon discovered that the results were

less satisfactory than when the samples were heated at 80° C. for 10 minutes in order to destroy other organisms which are less resistant or in non-spore form. The progress of putrefaction was much slower, when in evidence, and in fact a smaller percentage of positive results was obtained than by the heat method.

Numerous attempts were made to isolate the anaërobes. This was comparatively easy, as far as the organisms of the malignant edema bacillus type were concerned; with the putrificus bacillus, however, a great deal of difficulty was encountered, and in all but two attempts the results were unsatisfactory. It is indeed a difficult matter to obtain pure cultures of *B. putrificus* from any source.

Normal stools, therefore, contain anaërobes of the putrificus and malignant edema types, but the number of these organisms is very small, except perhaps in some exceptional cases. They exist in the intestines as spores, in this form resisting the unfavorable conditions which must obtain in the normal human intestine. In certain kinds of disturbances of the digestive tract it is highly probable that the anaërobes take advantage of the new conditions and develop to such an extent as to cause excessive putrefaction.

It is impossible to explain satisfactorily the suppressive influence that the human intestine exerts on the putrefying anaërobes. Bienstock claimed that it was due to the antagonistic action of *B. coli* and *B. lactis aërogenes* to the anaërobes. Tissier and Passini discredit this view, and along with other investigators hold that it is due to the acids that are produced from the sugars present, and to the natural protective action of the walls of the small intestine. That there is a certain antagonism of the colon bacillus to the anaërobes in question, my experiments have clearly shown, but I am forced to admit that this antagonism is not strong enough to explain the occurrence of so few of the putrefactive anaërobes in the normal human intestine.

I am fully convinced that the putrificus bacillus which was found in 20 per cent of the stools examined by me was the original *B. putrificus*, and not the paraputrificus bacillus which Bienstock described in his later publication. Tavel,¹ however, observed an anaërobe in certain cases of appendicitis which

¹ Tavel: *Centralbl. f. Bakt., etc.*, i, xxiii, p. 538, 1898.

seems to answer Bienstock's description of *paraputrificus*. This is Tavel's so-called *pseudotetanus bacillus*.

One other obligate anaërobe appeared to me to be of considerable interest, in connection with the above studies, namely, *B. aërogenes capsulatus* of Welch, or *B. enteritidis sporogenes* of Klein. Tests for this organism were made by the method generally used for its detection. The same quantities of fecal matter as mentioned before (*a*, *b* and *c*) were introduced into tubes containing 8 to 10 cc. of milk. After heating the tubes at 80° C. for 10 to 12 minutes, they were placed under anaërobic conditions and kept at incubator temperature for one to three days. Coagulation of the casein with vigorous gas production as shown by the whipped condition of the cream and coagulum were taken as direct evidence of the presence of the Welch bacillus. In ten cases where the reactions were positive, the rabbit test was made and in every instance but one (which probably failed on account of faulty manipulation) the striking phenomenon of gaseous distension of the animal was obtained. The results of the fermentation tests were as follows:

TABLE SHOWING THE PRESENCE OF SPORES OF *B. AEROGENES CAPSULATUS* IN NORMAL FECES.

Feces	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>a</i> (2mg.)	?	—	—	+	—	—	—	+	—	+	+	—	+	+	+	—
<i>b</i> (8 mg.)	+	+	+	+	—	+	—	+	—	+	+	+	+	+	—	—
<i>c</i> (32 mg.)	+	+	+	+	+	+	—	+	+	+	+	+	+	+	—	+

These results refer only to the spores of the organism. There seems to be considerable evidence that the bacillus is present as such in rather large numbers and that the spores are few as compared with the bacilli. Stained preparations of normal feces show but very few spores, as a rule.

In any work on intestinal fermentation and putrefaction the investigator is confronted with several difficulties. Perhaps the most serious of these is the lack of good methods of identifying, isolating and enumerating the anaërobes in question.

The chief points in the present paper may be summed up as follows:

1. Real putrefaction is the work of strict anaërobes only.

2. *Bacillus tetani* has very little or no putrefactive action on native proteids.

3. The bacillus of malignant edema and of symptomatic anthrax have similar morphological and biochemical properties, and aside from their specific pathogenic action in the lower animals, offer a difficult problem of differentiation.

4. *B. aërogenes capsulatus* is primarily a fermentative organism. It has the ability often to attack native proteids to a slight degree, but the transformation is not one of genuine putrefaction.

5. *B. putrificus* and *B. maligniædematis* are present in normal feces, but only in a limited degree and in all probability in spore form only. As spores they are able to resist the unfavorable conditions of the human intestine. A knowledge of the relative numbers of putrefactive anaërobes in the intestine of normal individuals and those suffering from certain kinds of intestinal disturbances is of the greatest importance.

ON THE PRODUCTION OF GLYCOSURIA IN RABBITS BY THE INTRAVENOUS INJECTION OF SEA-WATER MADE ISOTONIC WITH THE BLOOD.

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Glycosuria produced in animals by the introduction into the circulation of various salt solutions, has been known for a number of years. As far back as 1871, Bock and Hoffmann¹ showed that the rapid injection of large quantities of a 1 per cent solution of sodium chloride was followed by the appearance of sugar in the urine. Later, Külz² used the acetate, carbonate, valerianate, and succinate of sodium, with similar results. Kessler³ also noticed glycosuria in cats after the injection of sodium carbonate. Fischer⁴ and Brown,⁵ working independently, confirmed the results of other investigators, and added the fact that calcium chloride inhibited the glycosuria thus produced. It should also be mentioned that MacCallum⁶ found sugar in the intestinal juice after the injection of considerable quantities of $\frac{N}{6}$ sodium chloride solution.

In a paper published recently in the *Biological Bulletin*⁷ I have shown that isotonic sea-water is a balanced solution for the heart of the turtle, in that such a heart will preserve its contractility for long periods when immersed in $\frac{N}{6}$ sea-water, and will "recover" after sodium chloride arrest, as well as it does in Ringer's solu-

¹ Bock, C., and Hoffman, F. A.: *Arch. f. Anat. u. Physiol.*, p. 550, 1871.

² Külz, E.: Quoted from Pflüger's *Glycogen*, p. 390.

³ Kessler: Quoted by O. H. Brown, *Amer. Journ. of Physiol.*, x, p. 378, 1904.

⁴ Fischer, M. H.: *University of California Publications*, i, p. 87.

⁵ Brown, O. H.: *loc. cit.*

⁶ MacCallum, John Bruce: *University of Calif. Publications*, i, p. 125, 1904.

⁷ Burnett, T. C.: *Biological Bulletin*, xiii, p. 203, 1907.

tion. It occurred to me to try if isotonic sea-water would or would not produce glycosuria if injected into rabbits. I had an idea that as sea-water contained calcium, no glycosuria would follow its injection; especially as Quinton¹ had injected large quantities into dogs without serious consequences (he mentions albumin, but says nothing about sugar). My first experiment however, proved me to be in error. Glycosuria has followed the injection of isotonic sea-water in every instance, and it is as well marked as that caused by sodium chloride.

The method employed was essentially that of Fischer, and the reader is referred to his article for its description. The rabbits were fed only on barley and hay. The experiments were rarely carried on for longer than four hours, and the amount of solution injected did not exceed one liter. It was assumed that if glycosuria appeared at all, it would manifest itself within these limits of time and quantity. No anesthetic of any kind was given. The animals were fastened on their backs to the rabbit board, and remained perfectly quiet, not seeming to suffer in the least from the injections, which were always into the marginal vein of the ear. In the males the urine was obtained by catheter; in the females by gentle pressure over the bladder. Only qualitative estimations of sugar were made with Fehling's solution, care being taken to precipitate the albumen, which generally made its appearance before the sugar.

The following tables will give the details of the results obtained:

TABLE I.

Experiment April 26, 1907. Belgian hare, male, wt. 2200 gms. Injection fluid, $\frac{M}{8}$ sea-water.

Time	Amount injected cc.	Amount of urine cc.	
10.10 a.m.			Began injection
10.25	35		
10.40	115	25	No sugar
10.55	100	100	" "
11.10	150	110	Trace sugar
11.25	150	120	Sugar
11.40	150	100	"
11.55	125	80	"
12.10 p.m.	140	110	Much sugar
Total.	965	645	

¹ Quinton, R.: *L'Eau de Mer Milieu Organique*, p. 165.

TABLE II.

Experiment May 4, 1907. Belgian hare, male, wt. 2100 gms. Starved for 5 days. Injection fluid, $\frac{M}{8}$ sea-water.

Time.	Amount injected cc.	Amount of urine cc.	
10.45 a. m. ..		15	No sugar. Began injection.
11.00 ..	50	5	" "
11.15 ..	50	15	" "
11.30 ..	60	40	" "
11.45 ..	65	50	Trace sugar.
12.00 ..	75	60	Sugar.
12.15 p. m. ..	75	74	"
12.30 ..	75	68	"
12.45 ..	75	30	"
1.30 ..	200	140	"
1.45 ..	75	60	"
Total.....	800	557	

In this case the reflexes were abolished, and it was thought the animal was dying. When removed from the board he was completely relaxed; unable to move. At 2.25 p. m. he assumed his natural position, but with the forelegs spread. From this time on he kept improving, and by 8 p. m. had entirely recovered.

It then became of interest to know what constituent of the sea-water was responsible for this effect. Obviously the best way to get at it was by excluding one at a time the various salts, and observing the effect. The first solution tried was Ringer's, which is practically sea-water, minus magnesium. Six experiments were made in all. In two instances no sugar appeared. In the other four traces of sugar appeared after from one to two hours. It was very transitory and never in sufficient amount to ascertain its percentage by titration. I am inclined to think that the presence of traces of sugar in these instances may be due to lack of oxygen. The large amount of solution injected must necessarily dilute the blood considerably, and hence fewer corpuscles come in contact with the inspired air. This is well shown in an experiment made May 29, 1907. After one hour and a quarter, during which 400 cc. of Ringer's solution were injected into a rabbit weighing about 1600 grams, and only 102 cc. of urine excreted, the lungs became œdematous, as evidenced by coarse rales, the inspiration was labored, and there was some cyanosis. Coincidentally a trace of sugar appeared in the urine. The quantity injected was then diminished to 265 cc. during the next hour,

with practically no increase in the amount of sugar. During the next hour only 110 cc. were injected, mostly during the first half hour, and fifteen minutes after the injection was stopped the urine was entirely free from sugar. However this is but a surmise and has not been verified by experiment. Leaving aside the question of sugar or no sugar, the difference between the action of the two solutions, sea-water and Ringer, is very marked. Unfortunately quantitative estimations were not made, but it is characteristic that the glycosuria by sea-water increases until there is a dense precipitate, with complete decolorization of the Fehling's solution certainly 0.2 per cent or more, whereas when Ringer is injected, the color of the Fehling is hardly changed, a slight precipitate on cooling being all. Also the glycosuria does not tend to increase in amount, and disappears quite promptly when the injection is stopped. It is quite different when magnesium is added to Ringer in the proportion in which it occurs in sea-water. Here there seems to be no difference between the glycosuria induced by sea-water and that induced by Ringer plus magnesium. Both increase in quantity during injection, both become very marked, and both persist for some time after the injection has ceased (in one case a trace of sugar was found after twenty-four hours). The following tables will illustrate.

TABLE III.

Experiment September 27, 1907. Belgian hare, male, wt. 1500 gms. Injection fluid, Ringer's solution.

Time.	Amount injected cc.	Amount of urine cc.	
8.50 a.m.		20	No sugar. Began injection.
9.05	50		" "
9.20	75	30	" "
9.35	75	40	" "
9.50	75	50	Trace sugar.
10.05	75	56	No "
10.20	75	72	" "
10.35	100	80	Trace Sugar
10.50	100	88	" "
11.05	75	80	" "
11.20	50	60	" "
11.35	50	32	" " Stopped injection.
11.50		55	" "
12.05 p.m.		25	" " Slight precipitate on cooling.
Total.	800	698	

TABLE IV.

Experiment May 27, 1907. Belgian hare, male, wt. 1400 gms. Injection fluid, Ringer 100 cc. + $MgCl_2$ 12 cc.

Time.	Amount injected cc.	Amount of urine cc.	
9.15 a. m....		38	No sugar. Began injection.
9.30 ...	50	12	" "
9.45 ...	75	30	" "
10.00 ...	75	50	" "
10.15 ...	90	60	" "
10.30 ...	60	40	Trace "
11.00 ...	150	112	Sugar. Increasing.
11.30 ...	125	118	" "
12.00 ...	150	115	" " Stopped injection.
12.15 p.m.		40	" " Heavy precipitate.
Total.....	775	615	

A solution consisting of 975 cc. of $\frac{M}{8}$ sodium chloride + 25 cc. of $\frac{3M}{8}$ calcium chloride + 116 cc. of $\frac{M}{8}$ magnesium chloride, was then tried. It will be remembered that Fischer was able to inhibit glycosuria by sodium chloride plus calcium chloride in the above proportions. The addition of the magnesium caused glycosuria to appear in one hour and a quarter, becoming very marked during the next hour, and persisting after the injections had been stopped. The experiment is tabulated below.

TABLE V.

Experiment September 28, 1907. Belgian hare, female, wt. about 1500 gms. Injection fluid, 975 cc. $NaCl$, 25 cc. $CaCl_2$, 116 cc. $MgCl_2$.

Time.	Amount injected.	Amount of urine.	
9.20 a. m....			Began injection.
9.35 ...	50		
9.50 ...	75	24	No sugar
10.05 ...	75	26	" "
10.20 ...	75	38	" "
10.35 ...	100	38	Trace sugar
10.50 ...	100	34	" "
11.05 ...	100	60	Sugar. More abundant.
11.20 ...	100	80	" "
11.50 ...		36	Discontinued.
			Sugar. Heavy precipitate.
Total.....	675	336	

It thus seems evident that the magnesium in sea-water is responsible for the glycosuria which follows its injection. It is possible we are dealing here with another instance of the antagonistic action between calcium and magnesium, pointed out by Loeb¹ in 1906.

The bulk of this work was done in the spring of 1907, previous to the summer vacation, and the results were mentioned by Dr. Loeb² in July last. There are other facts of interest that have been brought out during these investigations, which may form the subject of a future communication.

¹ Loeb, J.: *This Journal*, i, p. 427, 1906.

² Loeb, J.: *Biochem. Zeitschr.*, v, p. 353, 1907.

THE OXIDATION OF LEUCIN, α -AMIDO-ISOVALERIC ACID AND OF α -AMIDO-*n*-VALERIC ACID WITH HYDROGEN PEROXIDE.

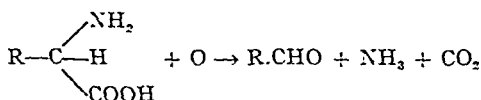
By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

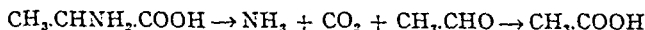
(Received for publication, December 7, 1907)

In a previous paper¹ an account was given of the oxidation of leucin, alanin and glycocoll with hydrogen peroxide in the presence of traces of ferrous sulphate and later the reaction was extended to a few other substances, such as sarcosin, creatin and creatinin.²

It was found that in general amido acids of the typical formula $R.CH.NH_2.COOH$ underwent oxidation so as to yield ammonia, carbon dioxide and an aldehyde, $R.CHO$, the latter being more or less completely oxidized to the corresponding acid according to the conditions of the experiment:



Thus alanin on oxidation furnished acetaldehyde, acetic acid, carbon dioxide and ammonia:

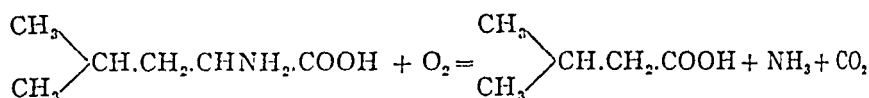


This type of reaction appeared to have considerable biological significance, since the removal of ammonia or carbon dioxide, or more commonly both together, from amino-acid molecules

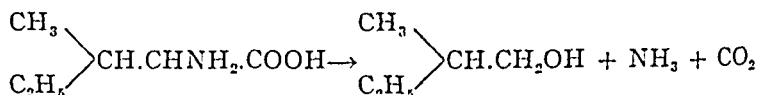
¹ This *Journal*, i, p. 171, 1906.

² *Ibid.*, i, p. 271, 1906.

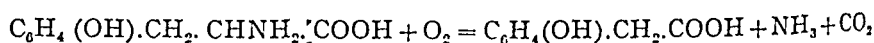
is a type of change frequently brought about by biological agencies. Thus the formation of isovaleric acid from leucin by putrefactive organisms was observed by Nencki:¹



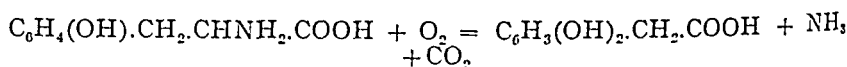
F. Ehrlich demonstrated the formation of a number of alcohols by the action of yeast upon the corresponding amido-acid. Thus optically active amyl alcohol² is formed from isoleucin as follows:



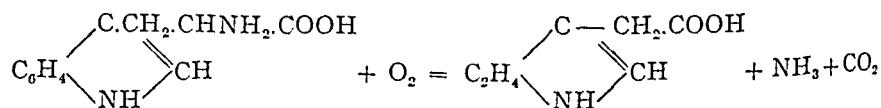
Other well-known examples of this type of reaction are found in the conversion of phenylalanin into phenylacetic acid by aerobic organisms³ and a similar formation of para-oxy-phenylacetic acid from phenylalanin and tyrosin.



In the animal organism a similar change is found in the formation of homogentisic acid from phenylalanin and tyrosin.



The production of indolacetic acid from tryptophan by putrefactive organisms must also be mentioned.⁴



Many other examples of this kind of biological change could be furnished but sufficient have been mentioned to show that this type of reaction is of common occurrence.

¹ *Gesammelte Arbeiten*, i, p. 204.

² *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 1027, 1907.

³ E. Schulze: *Zeitschr. f. physiol. Chem.*, vii, p. 202.

⁴ Hopkins and Cole: *Journ. of Physiol.*, xxix, p. 451, 1903.

It was therefore of interest to extend the previous investigations of the oxidation of amino-acids with hydrogen peroxide in order to try to simulate *in vitro* some of the reactions known to occur *in vivo*.

OXIDATION OF LEUCIN.

Previous experiments already published upon the oxidation of leucin with hydrogen peroxide, showed that the products were isovaleric aldehyde, isovaleric acid, ammonia and carbon dioxide. A small quantity of a substance giving the iodoform reaction was also detected but its identification was deferred pending further experiments; it has since been proved to be acetone. Some time after these results were published a paper appeared by F. Breinl and Oskar Baudisch¹ primarily upon the oxidation of keratin with hydrogen peroxide and incidentally a few observations were made upon the action of this reagent upon a few amino-acids including leucin. The authors referred to obtained a substance which they believed to be butyric aldehyde, and in support of this belief they refer to the old observation of Liebig,² who is stated to have obtained butyric aldehyde by the oxidation of leucin. These results might well be construed so as to throw doubt upon the correctness of my previous results. Accordingly further experiments have been made which completely confirm the original statements and incidentally it has been possible to show that the product of the oxidation of leucin with lead peroxide as described by Liebig is not butyric aldehyde but isovaleric aldehyde (p. 74).³

¹ *Zeitschr. f. physiol. Chem.*, lii, p. 159, 1907.

² Liebig's *Ann. d. Chem.*, lxx, p. 313.

³ Liebig states that his product was identical with Guckelberger's aldehyde obtained by the oxidation of proteins with chromic acid or manganese peroxide and sulphuric acid (*Ann. d. Chem.*, lxiv, p. 522). This substance has appeared in the literature as normal butyric aldehyde. It is however much more probable that it is isobutyric aldehyde derived from the breakdown of α -amido-isovaleric acid and probably of leucin. Incidentally it is of interest to note that Liebig's original statement is based upon an analysis of the aldehyde itself and that he actually found somewhat too high percentages of carbon and hydrogen. (Found: Carbon = 67.3 per cent; hydrogen = 11.4 per cent. Calculated for C_4H_8O : Carbon = 66.7 per cent; hydrogen = 11.1 per cent.) It is probable that Liebig's leucin contained a certain amount of α -amido-isovaleric acid

Breinl and Baudisch make no mention in the account of their experiments of the formation of isovaleric acid or other lower acids or of acetone, all of which must have been formed under the conditions of oxidation selected by them.

Identification of isovaleric aldehyde. Optically active leucin ($\frac{1}{10}$ gm. mol.) was slowly distilled with 3 per cent hydrogen peroxide ($\frac{1}{10}$ gm. mol.) to which a few drops of ammonia had been added. An oily distillate was obtained which smelt strongly of isovaleric aldehyde and gave all the usual aldehyde reactions. The distillate was acidified and redistilled to remove ammonia. The distillate so obtained gave a barely perceptible iodoform reaction. The aldehyde was converted into the sparingly soluble crystalline paranitrophenylhydrazone by precipitation with excess of nitrophenylhydrazine dissolved in 20 parts of 50 per cent acetic acid. The product was twice crystallized from alcohol and then was obtained in the form of fine dark yellow needles melting at 109° . For comparison the paranitrophenylhydrazones of isovaleric aldehyde, isobutyric aldehyde and *n*-butyric aldehyde were prepared from the pure aldehydes.¹

Isovaleric aldehyde-paranitrophenylhydrazone melts at 109°

Isobutyric	"	"	"	"	131.5° to 132°
<i>n</i> -Butyric	"	"	"	"	91° to 92°

There could be no doubt from the above results that the substance obtained from the oxidation of leucin was isovaleric aldehyde. For further confirmation, a portion of the aldehyde was oxidized with ammoniacal silver to isovaleric acid which was successively converted into the calcium and silver salts. On analysis

0.1634 gm. salt gave 0.0845 gm. silver = 51.71 per cent Ag
 $C_5H_8O_2Ag$ requires 51.67 " "

since the occurrence of this substance along with leucin among the amino-acids derivable from proteins was not discovered until a much later period. If this were so, Liebig's oxidation product would be a mixture of isobutyric aldehyde (see p. 75) and isovaleric aldehyde and thus the results of elementary analysis would be readily accounted for.

¹An account of the preparation and properties of these and other nitrophenylhydrazones will appear later.

On carrying out the oxidation as described with equimolecular proportions of leucin and hydrogen peroxide much leucin remains unattacked. The best yield of aldehyde is obtained when leucin is successively distilled with 5 to 6 molecular proportions of the peroxide divided into three separate portions, but under these circumstances much acetone is also formed (see below) and this interferes with the satisfactory identification of the aldehyde. When the reaction is carried out at a fairly high temperature as in these experiments, the use of ferrous sulphate as a catalyst is unnecessary.

Identification of acetone. Leucin ($\frac{1}{30}$ gm. mol. = 2.62 gm.) was distilled as before with $\frac{2}{30}$ gm. mols. of slightly ammoniacal 3 per cent hydrogen peroxide. After two-thirds of the liquid had been distilled, the process was twice repeated with equal additions of fresh hydrogen peroxide. The aldehyde in the combined distillates was then oxidized to the corresponding acid by warming with Tollen's ammoniacal silver solution containing caustic soda. The liquid was warmed to about 50° and then allowed to stand over night. The precipitated silver was then filtered off and the filtrate almost but not quite neutralized with phosphoric acid. The liquid was then distilled and the first part of the distillate readily gave all the usual reactions for acetone. For identification part of it was acidified with acetic acid and then treated with paranitrophenylhydrazine dissolved in 30 per cent acetic acid. The product was crystallized twice from alcohol and once from a mixture of benzene and petroleum. It melted at 147° to 149° and had all the properties of acetone-paranitrophenylhydrazone.

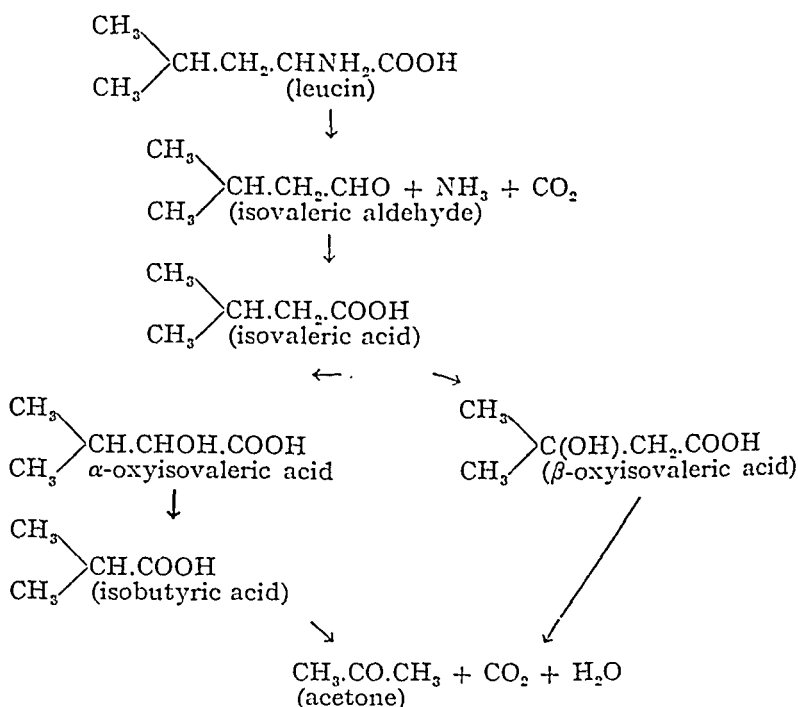
Formation of fatty acids. The residues from the various distillations of leucin with hydrogen peroxide were strongly acidified with phosphoric acid and redistilled. A considerable amount of volatile fatty acids was obtained in this way, corresponding to about 20 per cent of the leucin oxidized. The barium salts were first prepared and then the silver salt by precipitation with silver nitrate. On analysis

0.1709 gm. gave	0.0906 gm. silver	=	53.02 per cent Ag
$C_4H_7O_2Ag$ requires	51.67	"	"
$C_4H_7O_2Ag$ "	55.38	"	"

The results of the analysis show that the acid was not pure

isovaleric acid but that an acid of lower molecular weight was present. Special experiments have shown that ammonium isovalerate is readily oxidized by hydrogen peroxide and it is very probable that some isobutyric acid was formed as the result of the further breakdown of isovaleric acid initially formed from the leucin.

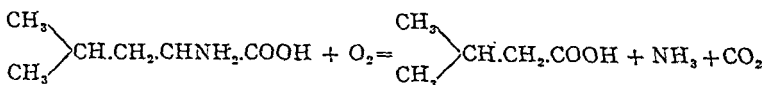
Mechanism of the reaction. The products obtained by the oxidation of leucin under the conditions described comprise isovaleric aldehyde, isovaleric acid, acetone, ammonia, carbon dioxide and probably isobutyric acid. It is of interest to trace the successive steps in the process of oxidation and the following scheme represents what is believed to be the most probable course of the reaction.



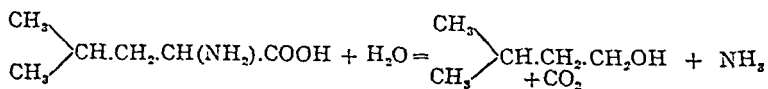
The initial production of isovaleric aldehyde, ammonia and carbon dioxide and the further oxidation of the first substance to isovaleric acid is readily understood. The formation of acetone offers several possibilities. In the first place the isovaleric acid may undergo oxidation at the α -carbon atom with formation

of isobutyric acid, α -oxyisobutyric acid being a hypothetical intermediate product. The isobutyric acid on further oxidation yielded acetone and carbon dioxide. That this change can be brought about by hydrogen peroxide was proved by special experiments in which acetone was separated from the products of oxidation of ammonium isobutyrate. On the other hand the isovaleric acid may undergo oxidation at the β -carbon atom with direct formation of acetone by the further oxidation of the hypothetical intermediary β -oxyisovaleric acid. It is not improbable that both of these modes of oxidation of isovaleric acid may occur, for it has been shown that butyric acid on oxidation under similar conditions undergoes oxidation at both α - and β -carbon atoms. Further light upon this question will be obtained from a careful study of the oxidation of ammonium isovalerate with hydrogen peroxide.

Biological significance. The oxidation of leucin with formation of isovaleric aldehyde, isovaleric acid and acetone presents analogies with several biological reactions. For example Nencki¹ showed that leucin was converted into isovaleric acid by the action of putrefactive organisms. The similarity between this change and the process of oxidation of leucin with peroxide is obvious.



Felix Ehrlich² in his striking investigations upon the origin of the amyl alcohols showed that these substances were derived from leucin and isoleucin by the action of living yeast cells. The formation of iso-amyl alcohol from leucin takes place in accordance with the following equation:



Ehrlich considers it probable that isovaleric aldehyde is the primary product of the breakdown of the leucin and this is then

¹ Nencki: *Gesammelte Arbeiten*, i, p. 202.

² *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 1027, 1907.

reduced to the corresponding alcohol. In addition it may be mentioned that both isovaleric aldehyde¹ and isovaleric acid² are formed during alcoholic fermentation and both doubtless originate from leucin. There is therefore a close similarity between the formation of these products from leucin and of those derived by oxidation with hydrogen peroxide.

The artificial production of acetone from leucin (and from isovaleric acid) by oxidation with hydrogen peroxide is of interest since the formation of acetone from these substances has been shown to occur in the animal body. Thus Embden, Salomon and Schmidt³ obtained acetone in considerable amounts by perfusing surviving livers with both leucin and ammonium isovalerate.

Leucin and isovaleric acid also lead to increased acetone excretion when given to diabetics⁴ or to persons placed upon a carbohydrate-free dietary.⁵ It may be that the exact mode of acetone production under these circumstances is not strictly comparable with its production by oxidation outside the body, as in the former case β -oxybutyric acid is believed to be an intermediate product in the reaction. It would be very desirable to determine if β -oxyvaleric acid is not produced at the same time, as this would bring the two types of reaction into still closer parallelism than now exists.

The foregoing biological reactions serve to emphasize the view that has been put forward, namely, that a close similarity exists between many biological oxidation processes and those capable of being effected under certain conditions by hydrogen peroxide.

OXIDATION OF α -AMIDO-ISOVALERIC ACID.

Since this acid is an important member of the group of amino-acids derived from the proteins, it was of interest to study the

¹ *Zeitschr. f. Spiritusindustrie*, ii, p. 183, 1888.

² K. Windisch: Ueber die Zusammensetzung der Trinkbranntweisse, *Arbeiten a. d. kais. Gesundheitsamt*, Berlin, viii, 1892; also Kruis u. Rayman: *Mittheil. d. Versuchstation f. Spiritusindustrie in Prag*, 1895, Heft ii.

³ *Beitr. z. chem. Physiol. u. Path.*, viii, p. 129, 1906.

⁴ J. Baer and L. Blum: *Arch. f. exper. Path. u. Pharm.*, lv, p. 89, 1906.

⁵ Borchardt and F. Lange: *Beitr. z. chem. Physiol. u. Path.*, ix, p. 116,

products of its oxidation with hydrogen peroxide. The amino-acid was prepared by Clark and Fittig's¹ method by the action of ammonia upon α -bromoisovaleric acid in a sealed tube at 100° . The acid was isolated and purified by recrystallization in the usual way. The oxidation was carried out exactly as described in the case of leucin so that the details will not be repeated.

Identification of isobutyric aldehyde. 1.17 gram ($= \frac{1}{100}$ gm. mol.) was slowly distilled with 3 per cent hydrogen peroxide ($= \frac{3}{100}$ gm. mol.). An oily distillate was obtained which contained much aldehyde and ammonia. It was redistilled after acidification with phosphoric acid. Acetone was practically absent as judged by the iodoform reaction. The aldehyde was dissolved by addition of a little alcohol and then precipitated with excess of paranitrophenylhydrazine dissolved in 30 per cent acetic acid. The crystalline nitrophenylhydrazone was filtered off and the crude product melted at 129° to 130° . After a single crystallization from alcohol, the substance was obtained in the form of fine needles melting at 131° to 132° , in every way identical with the product prepared from pure isobutyric aldehyde.

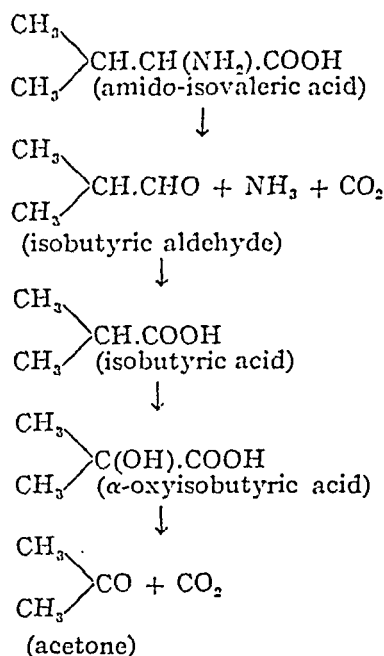
Identification of acetone. The oxidation was carried out as before with the difference that two distillations were carried out using in each case peroxide equivalent to $\frac{3}{100}$ gm. mol. The aldehyde in the distillate was oxidized by gently warming with Tollen's silver solution (2 gm. AgNO_3) and then the acetone was distilled off from the partly neutralized solution. The distillate gave the usual reactions for acetone including a strong iodoform reaction. It was definitely identified by converting it into the paranitrophenylhydrazone which after two recrystallizations from alcohol melted sharply at 149° .

Identification of isobutyric acid. The residues left after the aldehydes and acetone had been removed by distillation were acidified with phosphoric acid and redistilled. The acid was converted successively into calcium and silver salts. The latter crystallized from hot water in large plates characteristic of isobutyric acid. On analysis:

0.0831 gm.	gave 0.0463 gm. Ag	≈ 55.7 per cent Ag
	$\text{C}_4\text{H}_7\text{O}_2$ Ag requires	55.3 "

¹ *Ann. d. Chem.*, cxxxix, p. 200, 1866.

Mechanism of the reaction. The products of the oxidation of α -amido-isovaleric acid are found to be isobutyric aldehyde, isobutyric acid, acetone, ammonia and carbon dioxide. The formation of these substances can be readily followed from the following scheme:



The formation of isobutyric aldehyde and isobutyric acid, ammonia and carbon dioxide is an entirely similar change to that observed in case of other amino-acids. The acetone is a secondary product derived from the further oxidation of isobutyric acid, α -oxyisobutyric acid being a probable intermediate stage. Special experiments showed that the ammonium salts of both isobutyric acid and α -oxyisobutyric acid on oxidation with hydrogen peroxide yielded acetone in large quantity. In this respect the oxidation with peroxide differs from the usual process in the animal organism, since apparently neither isobutyric acid nor α -oxyisobutyric acid yield acetone by tissue oxidation. The first stage in the oxidation *in vitro*, involving the production of isobutyric aldehyde and isobutyric acid, closely simulates the biological process since α -amido-isovaleric acid in all probability is converted into isobutyric acid in the animal body, at any

rate its failure to produce acetone when administered to diabetics or when perfused through a surviving liver is most readily interpreted in this way.

The isobutyl alcohol in fusel oil doubtless originates from α -amido-isovaleric acid by a reaction entirely similar to the fermentative decomposition of leucin with production of isoamyl alcohol which has already been referred to. The probable intermediate production of isobutyl aldehyde is a reaction analogous to the oxidation of α -amido-isovaleric acid with hydrogen peroxide.

THE OXIDATION OF α -AMIDO-*n*-VALERIC ACID.

Although this substance is not a naturally occurring amino-acid, it was of interest to see if on oxidation it conformed to the general reaction already discussed, and as a result of the experiments it was found to behave in a perfectly normal manner. The oxidation was carried out under the same conditions as in the previous oxidation.

Identification of n-butyric aldehyde. α -Amido-*n*-valeric acid ($\frac{1}{10}$ gm. mol.) was slowly distilled with 3 per cent peroxide ($= \frac{1}{10}$ gm. mol.). The oily distillate contained much aldehyde and ammonia. It was redistilled after acidification. Acetone and acetaldehyde were practically absent as judged by the iodoform reaction. The aldehyde was converted into its paranitrophenylhydrazone in the usual way. After crystallization from alcohol it melted sharply at 91° to 92° and was identical with the product similarly prepared from pure normal butyric aldehyde.

Volatile acids. The residue remaining after the butyric aldehyde had been removed by distillation was acidified with phosphoric acid and redistilled. The volatile acids were converted into the calcium salts and then the silver salt. The acid present was mainly butyric acid but traces of formic acid could be detected and it was probable that traces of acetic acid and propionic acid were present since these acids are formed by the further action of hydrogen peroxide upon ammonium butyrate.¹ The analysis of the silver salt gave the following results:

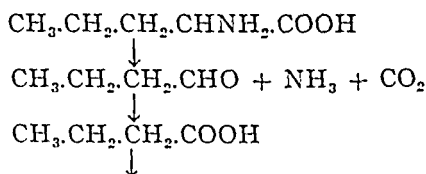
0.2050 gm. gave 0.1152 gm. silver = 56.2 per cent Ag
 $C_4H_7O_2Ag$ requires 55.4 " "

¹ This volume, p. 77.

The analysis corresponds with a salt mainly composed of silver butyrate with traces of acids of lower molecular weight.

The formation of acetaldehyde and acetone. Since the above results indicated that butyric acid was formed by the oxidation of α -amido-*n*-valeric acid and a previous investigation (p. 77) had shown that this substance on further oxidation yielded a variety of products including acetone and acetaldehyde, it was decided to try to detect these products among the products of more complete oxidation of α -amido-*n*-valeric acid. Accordingly 1.03 gram of the amino-acid was oxidized by successively distilling with three portions of 20 cc. of 3 per cent peroxide. The first distillate contained much butyric aldehyde but scarcely any acetone or acetaldehyde; the third, contained very decided amounts of acetone and acetaldehyde and practically no butyric aldehyde. The acetaldehyde was detected by Rimini's reaction with piperidin and sodium nitroprusside. The acetone was detected by the iodoform reaction in the distillate obtained after the aldehyde had been removed by oxidation with Tollen's silver solution at a gentle heat. The quantity was not large but amply sufficient to demonstrate its presence.

Mechanism of the reaction. The reaction had obviously proceeded according to the following scheme:



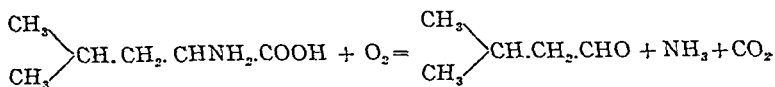
(Lower fatty acids, aldehyde, acetone and carbon dioxide according to scheme on p. 80.)

It would be of interest to try the fate of α -amido-*n*-valeric acid in the animal body under suitable conditions since it is extremely probable from the above results that excretion of the "acetone bodies" would be observed.

OXIDATION OF LEUCIN AND α -AMIDO-ISOVALERIC ACID WITH LEAD PEROXIDE (LIEBIG'S METHOD).

In the discussion of the oxidation products of leucin reasons were brought forward tending to throw doubt on the correctness of Liebig's statement that leucin on oxidation with lead peroxide

gave butyric aldehyde. By repeating the experiments it has been found that leucin yields isovaleric aldehyde instead of butyric aldehyde, while α -amido-isovaleric acid yields isobutyric aldehyde.



Lead peroxide was prepared by boiling red lead with dilute nitric acid and then thoroughly washing the product with hot water. The amino-acids were dissolved in water and distilled with excess of the peroxide. The product from leucin was in part converted into the paranitrophenylhydrazone which after crystallization from alcohol melted at 109° and was identical with the product prepared from pure isovaleric aldehyde. The remainder of the aldehyde was oxidized to isovaleric acid with ammoniacal silver solution. The isovaleric acid was separated by distillation and converted into calcium and silver salts. On analysis:

$$\begin{array}{rcl} 0.1089 \text{ gm. gave } 0.0563 \text{ gm. silver} & = & 51.70 \text{ per cent Ag} \\ \text{C}_5\text{H}_9\text{O}_2\text{Ag requires} & & 51.67 \text{ " "} \end{array}$$

α -Amido-isovaleric acid was oxidized in the same manner and the aldehyde in the distillate was converted into the paranitrophenylhydrazone which after crystallization melted sharply at 131° to 132° .

A study is being made of the oxidation of other types of amino-acids with hydrogen peroxide.

SUMMARY.

I. Leucin on oxidation with hydrogen peroxide yields primarily isovaleric aldehyde, isovaleric acid, ammonia and carbon dioxide. On further oxidation acetone is formed from the isovaleric acid. The reaction very closely resembles several of the biochemical changes in which leucin takes part.

II. Breinl and Baudisch's statement of the formation of isobutyric aldehyde by the oxidation of leucin is incorrect.

III. The product obtained by Liebig by the oxidation of

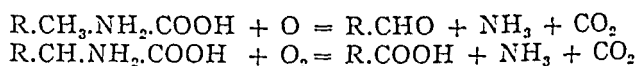
leucin with lead peroxide is not butyric aldehyde but isovaleric aldehyde.

IV. α -Amido-isovaleric acid, oxidized under similar conditions with hydrogen peroxide, yields isobutyric aldehyde, isobutyric acid, ammonia and carbon dioxide. A similar reaction occurs in some biochemical changes. Part of the isobutyric acid is oxidized further with production of acetone and carbon dioxide.

V. Oxidized with lead peroxide by Liebig's method, α -amido-isovaleric acid yields isobutyric aldehyde.

VI. α -Amido-*n*-valeric acid on oxidation yields primarily *n*-butyric aldehyde, butyric acid, ammonia and carbon dioxide. On further oxidation the butyric acid is in part decomposed with formation of acetone, aldehydes, lower fatty acids and carbon dioxide.

VII. The first steps in the oxidation of each of the above amino-acids resulting in the formation of aldehydes and acids are entirely analogous to the changes observed in the case of the oxidation of other amino-acids. The reaction may be expressed as follows:



A similar type of reaction is of common occurrence in many biochemical transformations.

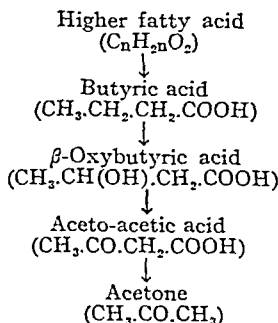
THE OXIDATION OF BUTYRIC ACID BY MEANS OF HYDROGEN PEROXIDE WITH FORMATION OF ACETONE, ALDEHYDES AND OTHER PRODUCTS.

By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, December 7, 1907.)

Among the numerous investigations made with the object of throwing light upon the mode of oxidation of organic acids in the animal organism few have been more suggestive than those concerned with the origin of the "acetone bodies" from the saturated fatty acids. It will be recalled that the acetonuria resulting from starvation, from a carbohydrate-free dietary, or from experimental or human diabetes is associated with an excessive fat catabolism. It is generally conceded that the long carbon chain in the molecule of the higher fatty acids is resolved in such a way as to yield one or more molecules of butyric acid and that the acetone bodies (β -oxybutyric acid, aceto-acetic acid and acetone) result from the further oxidation of this substance:



There is a mass of indirect experimental evidence to support this view of the origin of β -oxybutyric acid, aceto-acetic acid and

acetone from butyric acid, and of the latter from the higher fatty acids. Thus increased excretion of acetone and of the "acetone bodies" results from the injection into diabetic animals of butyric acid and of a number of substances, such as caproic acid, isovaleric acid and leucin, which can give rise to butyric acid in the course of their oxidation in the animal body. Similar results are obtained when the same substances are administered to persons in whom the normal processes of oxidation have been influenced either through starvation or consumption of a carbohydrate-free diet, or by diabetic disease.

If butyric acid be the precursor of β -oxybutyric acid, acetoacetic acid and acetone, it is necessary to assume that in the organism, the first stages of the oxidation of butyric acid, if not exclusively at least in part, take place at the β -carbon atom. Up to the present, however, it has not been possible to bring about a similar oxidation of butyric acid outside the body. Thus on prolonged boiling with nitric acid, butyric acid yields succinic acid;¹ with manganese dioxide and dilute sulphuric acid as oxidizing agent, propyl butyrate and ethyl butyrate are formed.² Chromic acid oxidizes butyric acid to acetic acid and carbon dioxide,³ while alkaline potassium permanganate brings about complete oxidation to carbon dioxide and water.⁴ The object of the present paper is to show that by a suitable choice of oxidizing agents, it is possible to oxidize butyric acid outside the body in a way that bears the closest resemblance to that believed to occur in the organism, and thereby to secure a confirmation of the results already arrived at from physiological studies.

Evidence is slowly accumulating which tends to show that oxidation in the β -position is not an uncommon primary process in the breakdown of many organic acids in the organism. Reference may be made to the interesting work of Knoop upon the break down in the organism of the aromatic derivatives of fatty acids, *e. g.*, phenylpropionic, phenylbutyric and phenylvaleric acids, in which the occurrence of primary oxidation in the β -position is shown to be very probable. But on reviewing the

¹ Dessaignes: *Ann. d. Chem.*, lxxiv, p. 361.

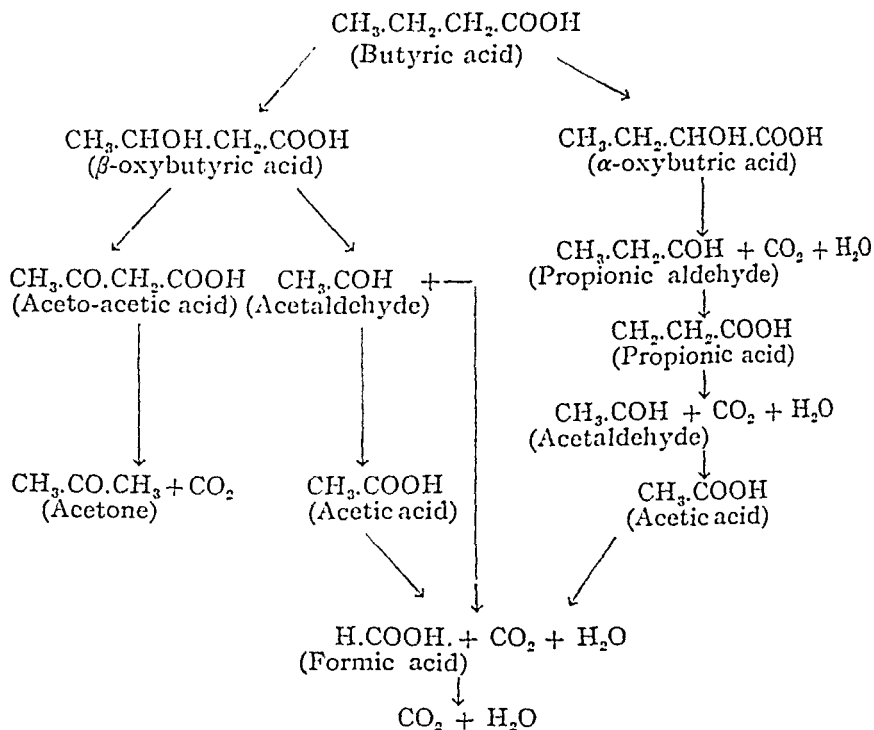
² Veill: *Ibid.*, cxlviii, p. 164.

³ Grünzweig and Hecht: *Ber. d. deutsch. chem. Gesellsch.*, xi, p. 1053.

⁴ Meyer: *Ann. d. Chem.*, ccix, p. 241.

experimental results of studies upon the oxidation of fatty acids by purely chemical means it will be found that comparatively few cases are to be found that furnish clear evidence of the β -carbon atom being the first point of attack.

The probability that oxidation of saturated fatty acids at the β -carbon atom is a common biological reaction makes it desirable to know more of the conditions under which such changes may be brought about outside the body. A number of previous observations had shown a remarkably close similarity between oxidations carried out with hydrogen peroxide acting under certain conditions and those occurring in the organism. It was therefore decided to study the action of peroxide of hydrogen upon butyric acid in order to see whether convincing evidence might not be forthcoming of the possibility of imitating outside the body those changes that are believed to progress within. As in previous experiments of this kind, neutral peroxide was allowed to act upon the ammonium salt of the acid. It was found that the ammonium butyrate was attacked to some extent even at the temperature of the air, but more readily on gently warming. The following products were detected: Aceto-acetic acid, acetone, propionic aldehyde, acetaldehyde, acetic acid, formic acid and carbon dioxide. Indirect evidence was obtained of the presence of propionic acid. From the fact that both aceto-acetic acid and acetone were formed, the latter substance in large quantity (see experimental part), it is very probable that β -oxybutyric acid was an intermediate product of oxidation and this view was confirmed by the fact that β -oxybutyric acid itself on oxidation with peroxide under similar conditions also gave acetone, acetaldehyde, acetic acid, formic acid and carbon dioxide (see following paper). From a consideration of the products of oxidation together with a knowledge of the further action of peroxide of hydrogen upon some of the intermediate products it is possible to draw a picture of the successive steps in the breakdown of butyric acid.

Oxidation of Butyric Acid with Hydrogen Peroxide.

It will be seen from the diagram that the initial breakdown of butyric acid appears to progress in two directions. It will be convenient to consider first that taking place with initial formation of β -oxybutyric acid. The β -oxybutyric acid undergoes further decomposition in two directions, yielding on the one hand aceto-acetic acid, which is then resolved into acetone and carbon dioxide, and on the other, acetaldehyde and probably other products which undergo further oxidation. The acetaldehyde in turn is oxidized to acetic acid and this eventually yields formic acid and carbon dioxide. The formic acid is lastly also oxidized to carbon dioxide and water. It is probable that several intermediate products are formed in the conversion of acetic acid into formic acid.¹ That acetic acid actually is oxidized to formic acid under the conditions prevailing in the oxidation of the butyric acid was proved by direct experiment. Hopkins and Cole² have investi-

¹ Cf. Dakin: *This Journal*, iii, p. 57.

² Hopkins and Cole: *Proc. Roy. Soc.*, lxxviii, p. 21.

gated the action of peroxide of hydrogen upon acetic acid under somewhat different conditions and obtained evidence of the formation of glyoxylic acid and formaldehyde in addition to formic acid. It is natural to assume that these substances were produced by the oxidation of the acetic acid formed during the oxidation of butyric acid, but to a large extent underwent further oxidation. No special attempt was made to isolate glycollic or glyoxylic acids, although qualitative reactions for the latter substance were obtained. No evidence was obtained of the presence of formaldehyde.

The presence of propionic aldehyde among the oxidation products of butyric acid indicates that part of the butyric acid underwent oxidation in a different way from that already indicated. The formation of this substance necessitates the assumption that oxidation at the α -carbon atom has taken place. It is natural to assume that α -oxybutyric acid is the primary product although this substance was not actually isolated. Identical products were obtained when α -oxybutyric acid was oxidized under similar conditions (see following paper). The propionic aldehyde on further oxidation yields propionic acid and the latter in turn yields acetic aldehyde and acetic acid. It is possible that lactic acid is an intermediate product in this reaction, but experiments to determine this point are at present lacking. Special experiments showed that acetaldehyde was actually formed by the action of hydrogen peroxide upon ammonium propionate. The acetic acid on further oxidation yields formic acid and eventually carbon dioxide and water as previously described for the products of oxidation of β -oxybutyric acid.

It will be seen that the earlier products of oxidation with the exception of acetone are all capable of further oxidation until they are eventually converted into carbon dioxide and water. Special experiments showed that acetone is very resistant to the oxidizing action of hydrogen peroxide and it is interesting to note that this is exactly comparable with the conditions believed to exist in the organism.¹ The difficulty of acetone combustion in the body has been demonstrated by Geelmuyden

¹ Wolfenstein has shown that acetone yields a peroxide on treatment with highly concentrated solutions of H_2O_2 .

while the easy tissue oxidation of the other products, such as propionic, acetic and formic acids, is well known.

The close similarity in many respects between tissue oxidation and oxidations affected by hydrogen peroxide has already been demonstrated¹ and in consequence it may be justifiable to try to draw some deductions from the results of the present investigation, as to the course of the breakdown of butyric acid in the body. It has long been a debatable question as to whether the breakdown of butyric acid with formation of the acetone bodies as intermediary products, as is known to take place under abnormal conditions, also represents the normal course of metabolism under physiological conditions. The difficulty of acetone combustion, even in the healthy organism, has been one of the main objections to the assumption that this substance is a product of normal intermediary metabolism. Notwithstanding this, the opinion just mentioned has been widely held, partly, no doubt, because hitherto it has been difficult to offer a satisfactory alternative theory of decomposition. In view of the already mentioned similarity between oxidation in the tissues and changes brought about by peroxide of hydrogen, is one not justified in assuming provisionally that butyric acid may break down in the body in more than one way, just as it does under the influence of peroxide of hydrogen? It may be that β -oxybutyric acid is the sole primary product and that this may break down so as to give either acetone, aceto-acetic acid being an intermediate product, or it may yield acetic and formic acids which then undergo further oxidation to carbon dioxide and water. The factors which determine which course of decomposition shall predominate have still to be determined. It must be conceded, too, that the possibility of α -oxidation of fatty acids in the organism is not entirely excluded and it may be that part of the butyric acid is broken down through the stage of propionic acid. The further complete breakdown of this substance in the organism presents no difficulty.

At any rate it appears from the results of the present investigation that there is at present no reason for assuming that acetone is necessarily a constant intermediate product of the tissue oxida-

¹ This *Journal*, i, pp. 171 and 271; iii, p. 419, and pending paper.

tion of butyric acid. It is probable that other modes of oxidation by way of acetic and formic acids (and possibly, although less likely, propionic acid) are at the disposal of the organism.

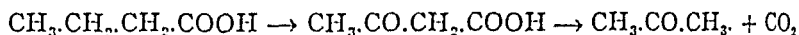
The demonstration of the easy formation of the acetone bodies by the direct oxidation of butyric acid renders superfluous the various hypotheses of the *synthetic* formation of these substances put forward at various times by von Jaksch, Geelmuyden, Magnus-Levy, Schwarz and others.

EXPERIMENTAL.

For the following experiments Kahlbaum's pure butyric acid was redistilled and the fraction boiling between 163.2° and 163.5° was employed. It was usually converted into the ammonium salt as required by addition of a slight excess of ammonia and then heated with neutral 3 per cent peroxide of hydrogen. In almost all the experiments it was so arranged that peroxide equivalent to two gram mols. was employed for the oxidation of every one gram mol. of the ammonium butyrate. Under these conditions a considerable amount of butyric acid remains unoxidized and there is opportunity for detecting some of the intermediate products of oxidation. The first point determined was the amount of carbon dioxide which was given off under these conditions.

Carbon dioxide. $\frac{1}{100}$ gm. mol. = 0.88 gm. butyric acid was neutralized with a slight excess of ammonia and the resulting solution boiled for a few minutes to expel any traces of carbon dioxide. The solution was then cooled and 3 per cent peroxide, equivalent to $\frac{2}{100}$ gm. mol. added. The mixture was contained in a flask placed in a water-bath and was connected with a series of absorption-bulbs all of which contained standard baryta solution with the exception of the first, which contained sulphuric acid to absorb any ammonia that might come over. A current of washed carbon-dioxide-free air was aspirated through the flask by means of a pump. The water-bath surrounding the flask was gradually heated, eventually to the boiling temperature. The current of air was maintained for almost three hours. The excess of baryta in the absorption-bulbs was determined by titration with $\frac{N}{2}$ sulphuric acid using phenolphthalein as

indicator.¹ In two experiments carbon dioxide equivalent to 0.1155 gm. and 0.1100 gm. was obtained. If the whole of this carbon dioxide had resulted from the decomposition of butyric acid with formation of acetone according to the equation:



the amount would be equivalent to about 26 per cent decomposition. By adding more peroxide of hydrogen to the residue in the flask and again neutralizing and warming, a further evolution of carbon dioxide almost as large as the initial amount may be obtained. If desired, the process may be repeated until the whole of the butyric acid is decomposed.

Non-volatile products. That the reaction had not proceeded simply with formation of acetone and carbon dioxide was proved by determining the mean molecular weight of the unoxidized residual fatty acids. It was found to be lower than that of butyric acid, showing that fatty acids of lower molecular weight had been formed. The residue from the oxidation of butyric acid with two mols. peroxide was made exactly neutral with ammonia and precipitated with excess of silver nitrate. The silver salt was analyzed:

0.8577 gm. gave	0.4883 gm. silver	= 56.93 per cent Ag	= M.W. 82.7
	$\text{C}_4\text{H}_7\text{O}_2\text{Ag}$ requires	55.36	" " and " 88

The non-volatile residues from a number of butyric acid oxidations were combined and evaporated to small bulk. An unsuccessful attempt was made to obtain evidence of the presence of β -oxybutyric acid by distilling the residue with strong sulphuric acid and examining the distillate for crotonic acid. The negative result does not, however, entirely preclude the possibility of its presence as its detection in small amount is rendered very difficult when accompanied by a large quantity of butyric acid.

Volatile products. The oxidation was carried out by very slowly distilling a mixture of ammonium butyrate (1 gm. mol.) and hydrogen peroxide (2 gm. mol.). The distillate was caught in a receiver placed in a freezing mixture. It smelt strongly of acetone and aldehydes and contained ammonia and some un-

¹ The liquid in the absorption-bulbs smelt strongly of acetone.

changed peroxide of hydrogen. The distillate was acidified with sulphuric acid and redistilled. The amount of acetone plus acetaldehyde was estimated by titration with standard iodine and calculated as acetone, and proved to be about 9 to 12 per cent of the amount of butyric acid taken for oxidation. But as less than 30 per cent of the butyric acid underwent oxidation the actual yield was about 40 per cent of the butyric acid oxidized, equivalent to about 50 per cent of the amount of acetone theoretically possible. Subsequent investigation showed that the proportion of acetone to aldehyde varied but was usually about 3:1.

The acetone was identified as follows. Distillates containing acetone and aldehydes were treated with ammoniacal silver solution together with some caustic soda. After gently warming and allowing to stand for some time the solution was filtered from precipitated silver and then almost but not quite neutralized with phosphoric acid. On distillation acetone passed over containing only traces of aldehydes. Part of the distillate was treated with paranitrophenylhydrazine acetate. An abundant precipitate of the hydrazone was at once obtained, which after recrystallization from dilute alcohol was obtained in the form of shining golden yellow needles melting at 149° . Bamberger and Sternitzki¹ give 148° to 148.5° as the melting point of acetone paranitrophenylhydrazone. Another portion of the distillate (15 cc.) containing approximately a decigram of acetone was shaken vigorously with 0.5 gram of benzaldehyde after addition of 3 cc. of a 10 per cent caustic soda solution. After 24 hours the somewhat pasty deposit of crystals was filtered off, washed with water and drained on a porous tile. It was then crystallized from alcohol. Well formed platelets of dibenzylidene-acetone melting sharply at 111.5° to 112.5° were readily obtained. The product dissolved with an orange-red color in strong sulphuric acid and with fuming nitric acid gave a deep red additive compound in the same manner as pure dibenzylidene-acetone prepared in other ways.

The preparation of the derivatives just described serves to demonstrate the presence of acetone convincingly, but several other reactions for acetone were carried out in addition. In every case a positive result was readily obtained. The tests used were

¹ *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 1306.

(i) the orthonitrobenzaldehyde condensation with formation of indigo; (ii) the nitroprusside reaction; (iii) Stock's reaction with hydroxylamine, etc.; (iv) the iodoform reaction.

The satisfactory identification of the aldehydes found in the distillate offered considerable difficulty owing to the presence of large amounts of acetone. The presence of aldehydes was easily demonstrated with ammoniacal silver solution, Fehling's or Schiff's reagent and acetaldehyde was readily detected by Rimini's reaction with sodium nitroprusside and piperidin. Formaldehyde could not be detected with the usual qualitative tests. The question of the presence of propionic aldehyde could not be directly settled, but indirect evidence was obtained as follows. Distillates containing a mixture of acetone and aldehydes were digested with an excess of Tollen's ammoniacal solution, containing caustic soda. At first the oxidation was carried out at the ordinary temperature of the air, afterwards in an incubator at 37° . After some hours the precipitated silver was filtered off and the solution acidified and distilled. In this way a distillate was obtained which contained volatile fatty acids corresponding to the aldehydes originally present. The fatty acid was found to contain acetic acid corresponding to the acetaldehyde already detected but in addition there was present a higher fatty acid, which proved to be propionic acid. This was shown by a determination of the mean molecular weight of the acid by means of the silver and barium salts. Three separate experiments gave the following results:

- I. 0.1711 gm. silver salt gave 0.1049 gm. Ag = 61.3 per cent Ag =
M. W. 69.1
- II. 0.1099 gm. silver salt gave 0.0674 gm. Ag = 61.3 per cent Ag =
M. W. 69.1
- III. 0.1718 gm. barium salt gave 0.1226 gm. BaCO_3 = 49.6 per cent Ba
= M. W. 70.6

The molecular weight was found to be intermediate between that of acetic acid (60) and propionic acid (72).

It was thought desirable to attempt the isolation of the propionic acid in order to furnish convincing evidence of the formation of the propionic aldehyde from which it was derived. Accordingly distillates containing the aldehydes and acetone from the oxidation of butyric acid, were distilled after the addition of a

little ammonia, in order to remove any possible traces of butyric acid which might be present. The distillate was then oxidized with Tollen's silver solution with very gentle warming. The precipitated silver was then filtered off and after neutralizing the bulk of the alkali with phosphoric acid the acetone was distilled off. On acidifying, the volatile acids were obtained by further distillation. They were converted into the barium salts and after evaporation the residue was stirred up with dilute alcohol. The residue was washed with more alcohol and in this way the bulk of the more soluble acetate was removed. The residue was dissolved in a little water and precipitated with excess of silver nitrate. The silver salt was dried *in vacuo* and on analysis proved to be silver propionate:

$$\begin{array}{rcl} 0.1622 \text{ gm. silver salt gave } 0.0972 \text{ gm. silver} & = & 59.9 \text{ per cent Ag} \\ \text{CH}_3\text{CH}_2\text{COOAg requires} & & 59.7 \quad \quad \quad \end{array}$$

Action of Hydrogen Peroxide upon Ammonium Butyrate at 40°.

I. *Detection of aceto-acetic acid.* Butyric acid (1 gm.) was neutralized with a very slight excess of ammonia and then 16 cc. of 3 per cent hydrogen peroxide was added. The whole was then incubated over night at 37°. The liquid was then shaken with pure charcoal to decompose the bulk of the excess of peroxide. The filtrate gave a strong reaction for aceto-acetic acid when tested for by the Arnold test with para-anidoacetophenone. The Bondy and Schwarz reaction with iodine was also obtained but the reaction with ferric chloride was largely obscured owing to the presence of large amounts of fatty acids. On warming the solution, the aceto-acetic acid readily decomposed with formation of acetone and carbon dioxide.

II. *Detection of acetone and acetaldehyde.* 8.8 grams of butyric acid ($\frac{1}{10}$ g. mol.) was neutralized with ammonia and $\frac{2}{10}$ g. mols. of 3 per cent hydrogen peroxide added. The mixture was placed in a tightly corked thick-walled flask and incubated over night at 37°. On shaking part of the fluid with either precipitated metallic silver or with charcoal so as to decompose the bulk of the excess of peroxide, it was found that the filtrate gave a strong iodoform reaction. This might, however, have been due to the aceto-acetic acid present in the liquid so that another method for the detection of acetone and aldehyde had to be devised. Accord-

ingly the liquid was distilled *in vacuo* at a temperature of not more than 40°. The distillate was caught in a receiver cooled with ice and salt. The distillate which contained ammonia and unchanged peroxide was acidified with phosphoric acid and redistilled. Aldehyde was readily detected in the distillate by the reactions with Tollen's silver solution, Schiff's reagent and by Rimini's reaction with sodium nitroprusside and piperidin. Part of the distillate was oxidized with Tollen's ammoniacal silver solution, then acidified and redistilled. Acetone was readily detected in the distillate with the iodoform reaction. In another series of experiments the distillation *in vacuo* was omitted and a current of air was bubbled through the liquid warmed to 40°. Aldehyde and acetone were readily volatilized and their presence detected by the tests previously employed.

SUMMARY.

Ammonium butyrate is readily oxidized by peroxide of hydrogen. The reaction progresses to a marked extent at low temperatures (37°) but is accelerated by warming.

The following products were detected: aceto-acetic acid, acetone, propionic aldehyde, acetaldehyde, acetic acid, formic acid and carbon dioxide. In addition propionic acid was probably present.

It is probable that α -oxybutyric acid and β -oxybutyric acid are the initial products of oxidation. With the exception of acetone, all the above products are capable of further oxidation with hydrogen peroxide until they are eventually converted into carbon dioxide and water. The probable course of the reaction is indicated diagrammatically on p. 80.

Acetone is very resistant to oxidation by hydrogen peroxide. A close similarity exists between the types of change involved in the oxidation of butyric acid with hydrogen peroxide and its oxidation in the body.

The view that there is no need to assume that the whole of the butyric acid formed in the body is oxidized with intermediate formation of acetone is supported. It is probable that β -oxybutyric acid may be decomposed so as to give acetic acid and its oxidation products. It is also possible, although

much less probable, that α -oxybutyric acid may be an alternative initial product of the tissue oxidation of butyric acid and this may be further oxidized through propionic acid and its subsequent oxidation products.

THE OXIDATION OF AMMONIUM SALTS OF HYDROXY-FATTY ACIDS WITH HYDROGEN PEROXIDE.

(GLYCOLLIC, LACTIC, α -OXYBUTYRIC, β -OXYBUTYRIC, α -OXYISOBUTYRIC, α -OXYISOVALERIC AND LEUCIC ACIDS.)

By H. D. DAKIN

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, December 7, 1907.)

In the preceding paper an account was given of the oxidation of ammonium butyrate with hydrogen peroxide and the results obtained was such as harmonized with the belief that α - and β -oxybutyric acids might be intermediate products of the reaction. This being so, it was of interest to investigate the action of hydrogen peroxide upon the ammonium salts of α - and β -oxybutyric acids, and in continuation to make a study of the oxidation products of several other oxy-acids.

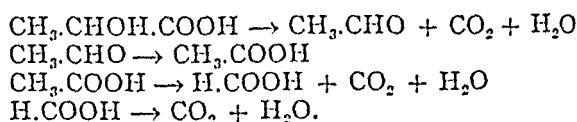
The ammonium salts of all the α -oxy-acids were found to undergo oxidation with hydrogen peroxide in a similar way, so that the reaction appears to be a general one. In each case with the exception of glycollic acid the oxy-acid yields carbon dioxide and either an aldehyde or ketone according to the structure of the acid.¹ In the cases where aldehydes result the corresponding saturated fatty acids are formed simultaneously by further oxidation. In some cases these fatty acids are capable of further oxidation.

¹ Glycollic acid on oxidation yields primarily glyoxylic acid and formaldehyde. Both these substances undergo further oxidation with peroxide of hydrogen yielding formic acid. The formic acid is also capable of further oxidation to carbon dioxide and water. The formation of glyoxylic acid by the oxidation of glycollic acid was described by the writer (this *Journal*, i, p. 271, 1906). The reaction had previously been discovered by Fenton and Jones (*Trans. Chem. Soc.*, lxxvii, p. 69, 1900), but much to my regret this paper was unfortunately overlooked.

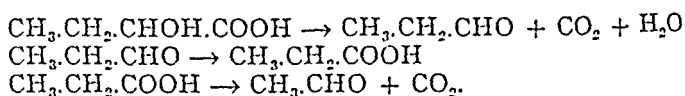
The initial changes may be represented as follows:



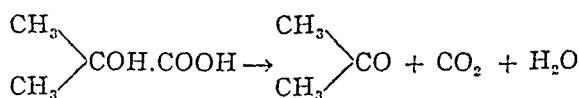
Thus lactic acid gives acetaldehyde, acetic acid and carbon dioxide.¹ Part of the acetic acid is oxidized further to formic acid and this in turn to carbon dioxide and water:



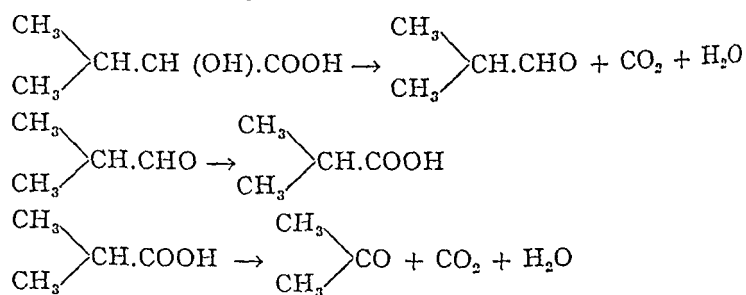
α -Oxybutyric acid yields propionic aldehyde and propionic acid. The latter in part undergoes further oxidation with production of acetaldehyde and acetic acid which are partially oxidized further as already indicated:



α -Oxyisobutyric acid, on the other hand, yields exclusively acetone and carbon dioxide. The acetone is incapable of further oxidation by the peroxide.

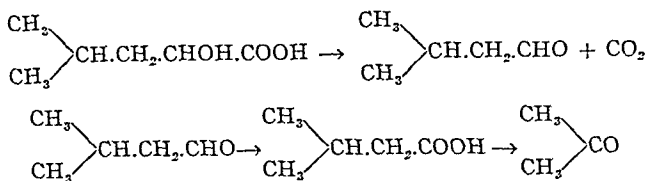


α -Oxyisovaleric acid primarily yields isobutyric aldehyde and isobutyric acid. The latter acid, however, undergoes further oxidation with acetone formation. Oxyisobutyric acid may possibly be an intermediate product:



¹ Fenton and Jones (*Trans. Chem. Soc.*, lxxvii, p. 69, 1900) investigated the action of hydrogen peroxide in the presence of iron salts upon free lactic acid at low temperatures. They found that pyruvic acid was formed and Hollemann has shown that this substance readily undergoes further oxidation with formation of acetic acid and carbon dioxide.

Leucic acid probably decomposes in an entirely similar fashion, forming isovaleric aldehyde and acid. The latter undergoes further oxidation with acetone production. Isobutyric acid or an oxyisovaleric acid are probably intermediary products, but further experiments are necessary to decide this point.



In most cases the primary change resulting in the conversion of the oxy-acid into an aldehyde (or ketone) is similar to that effected by other oxidizing agents than hydrogen peroxide. Thus Miller and Hofer¹ by the electrolysis of sodium salts of α -oxy-acids obtained aldehydes and acids. For example glycollic acid gave formaldehyde, formic acid and carbon dioxide, while α -oxybutyric acid gave propionic aldehyde and some formic acid. Kolbe² found that potassium lactate on electrolysis yielded acetaldehyde and carbon dioxide, while Liebig found that a similar change was brought about by manganese or lead peroxide and sulphuric acid. Ley³ obtained propionic aldehyde and propionic acid by oxidizing α -oxybutyric with chromic acid.⁴ Under the same condition α -oxyisovaleric acid gave isobutyric aldehyde and isobutyric acid.

The oxidation of β -oxybutyric acid, or rather of its ammonium salt, with hydrogen peroxide gave a number of oxidation products. The following were detected: aceto-acetic acid, acetone, acetic acid, formic acid and carbon dioxide. The initial stage appears to take place in two directions either with formation of aceto-acetic acid which undergoes further decomposition with

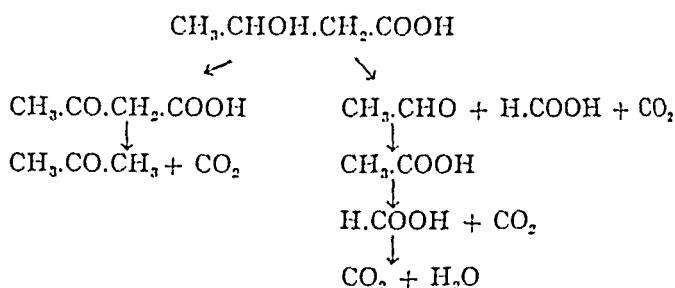
¹ *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 467.

² *Ann. d. Chem.*, cxiii, p. 244.

³ *Journ. Russ. Chem. Soc.*, ix, p. 131.

⁴ Markownikoff (*Ann. d. Chem.*, cliii, p. 309) believed acetic acid to be formed at the same time, but this was not confirmed by Ley.

production of acetone and carbon dioxide¹ or with formation of acetaldehyde and other products. The acetaldehyde on further oxidation yields acetic acid, formic acid and eventually carbon dioxide and water. The probable course of oxidation may be represented diagrammatically as follows:



All of the products of oxidation of the β -oxybutyric acids were obtained in the oxidation of ammonium butyrate by hydrogen peroxide, as described in the preceding paper. The results are therefore in harmony with the suggestion that β -oxybutyric acid is an intermediary product in the oxidation of ammonium butyrate with hydrogen peroxide.

The possibility of oxidizing β -oxybutyric acid to acetaldehyde and acetic acid and so furnishing an alternative method of decomposition to that involved in its oxidation to acetone and carbon dioxide, has a certain biological significance which is referred to in the preceding paper and so need not receive further mention. It is of interest to note that the reaction progresses to a marked extent at body temperature, but is much accelerated by warming. This is probably true of all the oxidations described in this paper.

Perhaps the most striking point in the results obtained by the oxidation of the oxy-acids is the fact that although decomposition of the oxy-acid undergoing oxidation is ordinarily far from complete, yet secondary changes resulting from the further oxidation of the primary oxidation products are continually in progress. This is due in part to the ease with which the ammonium salts of the saturated fatty acids undergo oxidation with hydrogen peroxide. It appeared to be of interest to obtain

¹ There is also the possibility of aceto-acetic acid undergoing further oxidation so as to yield acetic acid and other products. I intend to investigate this point.

some information of a roughly quantitative character as to the relative degree of oxidizability of the different oxy-acids investigated. Accordingly estimations were made of the carbon dioxide evolved under certain definite conditions of experiment in which varying proportions of hydrogen peroxide were employed. In each case $\frac{1}{100}$ gm. mol. of acid was oxidized in the form of its ammonium salt. If the oxidation were limited to the initial reaction involving the evolution of one molecule of carbon dioxide from each molecule of oxy-acid, the theoretical yield of carbon dioxide in each case would be 0.44 gram. This is, however, not strictly true for reasons which have already been mentioned, namely, the occurrence of further oxidation of initial products of oxidation. The results may be arranged in tabular form:

Acid oxidized. ($\frac{1}{100}$ g. mol.)	Quantity of 3 per cent H_2O_2 employed.	Carbon dioxide evolved.
Glycollic acid (0.76 g.)	1/100 g. mol.	0.19 gm.
" " " "	2/100 "	0.29 "
Lactic acid (0.9 g.)	1/100 "	0.15 "
" " " "	2/100 "	0.29 "
α -Oxybutyric acid (1.04 g.)	2/100 "	0.11 "
" " " "	3/100 "	0.15 "
β -Oxybutyric acid (1.04 g.)	1/100 "	0.06 "
" " " "	3/100 "	0.16 "
α -Oxyisobutyric acid (1.04 g.)	2/100 "	0.14 "
α -Oxyisovaleric acid (1.18 g.)	2/100 "	0.10 "

It will be seen that the lower members of the series are decidedly the most easily oxidized under the prevailing conditions. α - and β -oxybutyric acids show no marked differences among themselves although they appear to be oxidized slightly less readily than the oxyisobutyric acid. The differences are however not marked. The oxyisovaleric acid is somewhat less easily oxidized, the yield of carbon dioxide being about one-third that from glycollic or lactic acid under similar conditions of experiment. The method used for estimating the carbon dioxide evolved in the process of oxidation was the same as was employed in the preceding paper for the oxidation of butyric acid.

EXPERIMENTAL PART.

Glycollic acid. The oxidation of this substance with hydrogen peroxide has already been investigated.¹ It only remains to

¹ Dakin: *This Journal*, i, p. 271, 1906.

add that when the oxidation is carried out by warming the ammonium salt of the acid in faintly alkaline solution with hydrogen peroxide, the reaction is a vigorous one and practically all the glyoxylic acid which is regarded as the initial product is further oxidized to formic acid. Much of the latter undergoes further oxidation to carbon dioxide and water.

Lactic acid. Pure lactic acid ($\bar{1}^1_6$ gm. mol.) was neutralized with a slight excess of ammonia and then very slowly distilled with 2 gm. mol. of 3 per cent peroxide. The distillate was caught in a flask surrounded by a freezing mixture. It smelt strongly of acetaldehyde and gave all the usual reaction for that substance. The distillate which contained some ammonia and a little unchanged peroxide was redistilled after acidification with phosphoric acid. On treatment with paranitrophenylhydrazine acetate dissolved in 50 per cent acetic acid, an abundant precipitate of the hydrazone was at once obtained. The hydrazone melted at about 125° . After a single crystallization from a mixture of benzene and petroleum it melted sharply at 128° to 128.5° .¹

After the aldehyde had been removed by distillation, the residue in the flask was acidified with phosphoric acid and redistilled. The distillate contained both acetic and formic acids. The latter was removed by short boiling with chromic acid and the acetic acid again recovered by distillation. It was then converted successively into the barium and silver salts.

The latter was analyzed.

$$\begin{array}{rcl} 0.2868 \text{ gm. gave } 0.1861 \text{ gm. Ag} & = & 64.8 \text{ per cent Ag.} \\ \text{CH}_3\text{COOAg requires} & & 64.7 \quad " \quad " \end{array}$$

α -Oxybutyric acid. The acid was converted into the ammonium salt and oxidized with peroxide in the same way as was adopted in the case of lactic acid. The distillate smelt strongly of aldehyde and readily gave all the usual tests for these substances with Schiff's reagent, silver and copper solutions, etc. Propionic aldehyde was readily identified by preparing the paranitrophenylhydrazone. It was obtained in large quantity and after two crystallizations from alcohol it was obtained in the form of well-defined orange red needles melting sharply at 123° to 123.5° .

¹A description of the preparation and properties of this and other nitrophenylhydrazones will appear shortly.

Formaldehyde was absent but acetaldehyde was qualitatively detected by Rimini's reaction with sodium nitroprusside and piperidin. It was probably a secondary product derived from the oxidation of propionic aldehyde. Propionic aldehyde does not give Rimini's reaction but the products of its oxidation with peroxide contain acetaldehyde and readily give a strong reaction.

The acids formed on oxidation were liberated with phosphoric acid and recovered by distillation. Formic, acetic and propionic acid were present. The formic was removed by oxidation with chromic acid and the latter converted in the barium and silver salts. The analysis of the latter corresponded with a mixture of acetic and propionic acids.

0.2068 gm. gave 0.1308 gm. Ag.	=	63.3 per cent Ag.
$\text{CH}_3\text{CH}_2\text{COOAg}$ requires	59.7	" "
CH_3COOAg	64.8	" "

β -Oxybutyric acid. The oxidation was carried out as before by very slowly distilling a mixture of ammonium β -oxybutyrate ($\frac{1}{10}$ g.mol.) with 3 per cent hydrogen peroxide ($\frac{1}{10}$ gm. mol.). The distillate was acidified and redistilled. The combined yield of acetone and aldehyde as determined by iodine titration and calculated as acetone, corresponded to about 20 per cent of the theoretical amount. Acetone was identified by conversion into dibenzylidene-acetone, by shaking part of the distillate with benzaldehyde and a little caustic soda. After 24 hours the gummy precipitate was spread on a porous tile and subsequently crystallized from alcohol. The product was obtained in the form of fine platelets melting at 111.5 to 112.5°. The crystals dissolved in concentrated sulphuric acid to give an orange red solution. On treatment with concentrated nitric acid, a red insoluble product was obtained. Both of these reactions are characteristic of dibenzylidene-acetone.

Aldehydes were detected by the usual reagents. Formaldehyde was absent but the reactions for acetaldehyde were readily obtained, especially the test with piperidin and sodium nitroprusside.

To further characterize the acetaldehyde, part of the distillate was oxidized with Tollen's silver solution and the acid formed recovered by distillation. Acetic acid was identified by preparation of the barium and silver salts.

The volatile acids formed in the oxidation of the ammonium β -oxybutyrate were recovered by distillation as before. Formic acid was present in considerable quantity and was easily detected by the reaction with silver and mercury salts. After the formic acid had been destroyed by boiling with chromic acid, acetic acid was readily detected in addition.

Action of Hydrogen Peroxide upon Ammonium β -Oxybutyrate at 37°.

It was of interest to show that the oxidation of β -oxybutyric acid progresses at low temperatures as was shown to be the case with the oxidation of butyric acid (p. 87). Accordingly 1 gram of β -oxybutyric acid was neutralized with ammonia and digested in a closed flask for sixteen hours at 37° with 20 cc. of 3 per cent hydrogen peroxide. The liquid was then cooled and the bulk of the peroxide removed by agitation with freshly precipitated silver. Part of the liquid was tested for aceto-acetic acid with a positive result by the Arnold test with para-amidoacetophenone. The remainder was distilled *in vacuo* at a temperature of 40°. The receiver was kept in an efficient freezing mixture and the distillate was found to readily give the reactions for acetone and aldehyde which had previously served for their detection.

α -Oxyisobutyric acid. The oxidation was carried out as in the previous cases. The distillate as was expected gave no reactions for aldehydes but acetone was present in large amounts. The yield was about 30 per cent of the theoretical amount when 2 gm. mol. of 3 per cent hydrogen peroxide were used for the oxidation of 1 gm. mol. of ammonium α -oxyisobutyrate. The acetone was identified by conversion into acetone-paranitrophenylhydrazone. The product was crystallized from alcohol and melted sharply at 149°.

α -Oxyisovaleric acid. This acid was prepared by acting upon the sodium salt of α -bromoisovaleric acid with silver oxide. After boiling the liquid was acidified and the acid extracted with ether. On evaporation of the ether the acid readily crystallized. It was oxidized in the form of its ammonium salt with two gram mols. of 3 per cent hydrogen peroxide. The distillate was acidified with phosphoric acid and redistilled and was found to contain aldehydes and some acetone. The aldehyde was readily identified as isobutyric aldehyde by preparing the paranitro-

phenylhydrazone which after two crystallizations from alcohol melted sharply at 131° to 132° , corresponding with the same product prepared from isobutyric aldehyde. Another oxidation of α -oxyisovaleric acid was carried out in which four gram molecules of peroxide were used. The distillate contained much acetone, which was recognized by first removing the aldehydes by oxidation with Tollen's silver solution and then recovering the acetone by distillation and converting part of it into the paranitrophenylhydrazone which after recrystallization from alcohol, melted sharply at 149° to 149.5° . Another part was heated with benzaldehyde and caustic soda and gave dibenzylidene-acetone, which after crystallization from alcohol melted at 112° . The fatty acids formed in the oxidation of ammonium α -oxyisovalerate were recovered by distilling the acidified residue. It is a curious fact that distinct traces of formic acid were present. The formic acid was removed by warming with a little chromic acid and then the residual volatile acid was converted successively into barium and silver salts. The silver salt crystallized from hot water in plates characteristic of isobutyric acid.

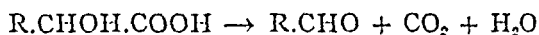
Analysis: 0.1499 gram gave 0.0835 g. silver = 55.6 per cent Ag
 $C_4H_7O_2$ requires 55.4 " "

Leucic acid. A small quantity of crude leucic acid, obtained by the action of nitrous acid upon natural leucin was oxidized under similar conditions to those in preceding experiments. Aldehydes were found in considerable quantity and acetone was also readily detected. The aldehydes appeared to be a mixture, but probably isovaleric aldehyde was the main constituent. By repeated crystallization a small quantity of the paranitrophenylhydrazone of the latter substance, melting at 109° to 110° , was obtained. The volatile acids found appeared to be a mixture of isovaleric and isobutyric acids. The reaction was not further investigated.

SUMMARY.

The products of the oxidation with hydrogen peroxide of the ammonium salts of glycollic, lactic, α -oxybutyric, β -oxybutyric, α -oxyisobutyric, α -oxyisovaleric acids have been studied. With the exception of glycollic and β -oxybutyric acids the initial reaction in each case results in the formation of an aldehyde

(in the case of α -oxyisobutyric acid acetone is formed) with liberation of carbon dioxide according to the following equation:



According to the conditions of the experiment, more or less of the aldehyde is oxidized to the corresponding acid. In some cases the acids are capable of further oxidation. Thus lactic acid yields acetaldehyde, acetic acid and carbon dioxide. Part of the acetic acid is further oxidized to formic acid if excess of peroxide be employed. Preliminary experiments with leucic acid indicated that it underwent oxidation in the same manner as the other oxy-acid.

Glycollic acid on oxidation yields primarily glyoxylic acid and formaldehyde and these on further oxidation yield formic acid which in turn is capable of further oxidation to carbon dioxide and water.

β -Oxybutyric acid on oxidation yields a variety of products including aceto-acetic acid, acetone, acetaldehyde, acetic acid, formic acid and carbon dioxide. The probable course of the reaction is indicated on p. 94.

It is anticipated that some of these reactions are analogous to some biochemical changes.

THE OCCURRENCE OF SKATOL IN THE HUMAN INTESTINE.

By C. A. HERTER.

(Received for publication, December 7, 1907.)

The color reaction between paradimethylamidobenzaldehyde (Ehrlich's aldehyde) and skatol¹ gives us an easy method of detecting the presence and of estimating the quantity of this putrefactive product. The employment of the method in routine and experimental work upon the digestive tract has led to the accumulation of numerous observations that have an interest both for the physiologist and the physician. It is my wish to bring together here the chief results of these observations.

It seems to be a general impression, at least among writers of text-books of physiological chemistry, that skatol is a regular product of intestinal putrefaction in the feces. Probably this impression is due to the statement made by Brieger to this effect²—a statement which requires some modification. Among healthy children under ten years of age I have found the occurrence of skatol in the feces to be quite exceptional whether they live on a milk diet or on a mixed diet inclusive of meat. Among adults also there are many who show no trace of skatol. It is, however, true that one frequently finds a trace of skatol in the freshly voided feces of healthy individuals.

Where there is an excessive degree of intestinal putrefaction there may be a marked increase in the skatol content of the feces. Whereas in persons who are normal (in the sense that they are

¹ On the Separation of Indol from Skatol and their Quantitative Determination, this *Journal*, ii, p. 267, 1906.

² "Das Skatol ist ein constantes Bestandtheil der menschlichen Excremente, fehlt aber in denen des Hundes." Ueber die flüchtige Bestandtheile der menschlichen Excremente. *Journ. f. prakt. Chem.*, xvii, p. 124. Reprinted in Nencki's *Opera Omnia*, i, p. 379.

unconscious of any disorder of digestion) one rarely finds more than 0.5 milligram of skatol in 100 grams of fresh feces, the quantity may reach 8 or 10 milligrams in persons who are the subjects of disturbed intestinal digestion. These quantities I have seldom observed and it may be safely stated that it is rare to find more than 5 milligrams in 100 grams, even where putrefactive decomposition is intense. In this respect the skatol content of the lower intestine differs from the indol content, which is sometimes greater than is represented by the above figures. Moreover it is true that among apparently normal individuals it is more common to find traces of indol in the feces than traces of skatol. To this rule there is observed an occasional exception in which skatol can be detected but not indol.

The detection of skatol in the feces in the absence of indol of course does not prove that there has been no production of indol in the gut, since it may have been formed at some level above the rectum and subsequently absorbed entirely. It is clear that this might be the case where we find skatol but no indol, despite the fact that indican is present in the urine and cannot be ascribed to any but an intestinal origin. The presence of skatol without indol in the lower part of the bowel might be ascribed either to a more ready absorption of indol than of skatol (assuming them to be produced in equal abundance at the same level), or to a relatively late production of skatol.

The following experiment was made with a view to determining whether the absorption of indol from the intestine is more rapid than that of skatol.

Into a loop of dog's ileum which had been previously washed out with salt solution there was introduced 100 cc. of a solution prepared by dissolving 5 mg. of indol and 5 mg. of skatol in 2 cc. of alcohol and diluting to 250 cc. with physiological salt solution. After the introduction of this fluid, the gut was returned into the abdominal cavity. The animal was killed at the end of 35 minutes, when the gut had in a large degree emptied itself. 26 cc. of fluid were found in the gut. After filtration this fluid was distilled and the distillate tested for skatol and indol by means of Ehrlich's aldehyde. By means of the color reaction obtained, it was possible to form a judgment as to the proportions of indol and skatol present in this fluid, a portion of the originally prepared solution being employed as a control in making this comparison. It was found that the tints obtained from the distillate from the gut were so nearly duplicated by

the tints obtained from the original solution that they could not be distinguished. Although there was this close correspondence in the colors obtained by the action of the aldehyde there was a less close correspondence between the colors obtained on shaking out with chloroform, the distillate from the fluid of the gut yielding a reddish rather than a purple tint, a result which may perhaps be regarded as pointing to the presence of slightly less skatol than indol. It was noticeable that the concentration of the indol and of the skatol in the fluid of the loop was less than one-quarter as great (judging by the intensity of the color reaction) as that of the original solution.

It is evident from the foregoing experiment that it gave no indication that indol is absorbed more readily than skatol—a result which harmonizes well with what is known of the close resemblance between indol and skatol in respect to solubility and chemical constitution. It thus appears in a high degree improbable that differences in the rate of absorption of indol and skatol from the intestine can account for the preponderance of skatol over indol that is sometimes noted in the contents of the human colon.

There is some evidence that skatol is in general a later product of putrefaction than indol. Nencki recommends long standing putrefactive mixtures containing muscle fiber and pancreatic gland, in order to obtain skatol in fair quantities.¹ I have many times noticed in the course of putrefactive experiments *in vitro* that skatol appeared several days later than indol. It is not certain that this fact helps to explain the finding of skatol at lower levels of the intestinal contents than indol, because the conditions of decomposition within the intestine are so different from those that are experimentally induced. Experiments made on human subjects with the aid of cathartics indicate that proteid food may yield skatol within 24 hours, whereas, on artificial culture media, I have never observed it before the lapse of several days. Still it is likely that although both indol and skatol are sometimes more rapidly formed in the intestinal tract than in experiments *in vitro*, the relatively later formation of skatol is also a feature here. This seems the most reasonable explanation of the occurrence of skatol in the feces without indol, in those cases where indol has certainly been formed and absorbed.

¹ Vortheilhafte Darstellung des Skatols, *Centralbl. f. d. med. Wiss.*, No. 47, p. 849, 1878; *Opera omnia*, p. 433.

As yet I have not had a sufficiently long experience to make a generalization with respect to the clinical conditions under which the intestinal contents persistently show the presence of skatol in excessive quantities. I have not found skatol abundant and persistent in the feces except in the case of persons who are ill or have recently been ill of some intestinal disorder. For this reason I have come to attach to its presence more significance than to the presence of indol, which is not infrequently found in the intestinal contents of persons in apparently good health, who are unconscious of any digestive disturbance. As to the kind of proteid food most apt to favor the production of skatol no definite statement can be made beyond the fact that a milk diet does not necessarily cause skatol production to cease, although it seems to render it less active than where the diet contains an equal quantity of nitrogen in the form of meat proteid.

Where skatol formation is active in the intestine there are usually other indications of excessive putrefaction, especially an increase in the ethereal sulphates of the urine and an excessive formation of hydrobilirubin within the gut. Indol production is usually excessive but I have observed instances in which this was not the case, instances in which the excessive putrefaction was skatolic rather than indolic in type. This is not usually a transitory phenomenon but is likely to be a long persistent feature.

The excessive formation of skatol I have found especially in what I have described as instances of chronic excessive saccharo-butyric intestinal putrefaction. Mental or emotional depression has in some of these cases been the most persistent clinical feature; in others there has been a moderate or considerable degree of simple anæmia. In still others there have been present the blood changes characteristic of pernicious anæmia. I have come to believe that the chronic digestive disturbances of pernicious anæmia are almost regularly associated with an excess of skatol in the feces. In a case of appendicitis which came under my observation the feces contained skatol for several weeks after an operation for removal of the appendix; with return to health this substance gradually disappeared. In a patient with multiple neuritis associated with persistent constipation and great production of intestinal gases, skatol was regularly obtained in abundance from the feces during the period of paralysis. With gradual convales-

cence there was a complete disappearance of the skatol of the feces. Several other examples could be mentioned which illustrate the disappearance of skatol from the feces, concomitantly with a betterment in clinical conditions. Apparently it would be worth while to make careful systematic observations on the quantities of skatol obtainable from intestinal material derived from a variety of patients suffering from intestinal disease, with a view to learning in how far the presence of this substance is a guide to the intensity and course of bacterial processes in the intestinal tract.

In practice I have found it convenient to make use of the following mode of procedure in examining human feces for skatol. Twenty grams of fresh material are ground in a mortar with a convenient quantity of water. The suspension is then diluted up to 300 cc. It is now acidified with phosphoric acid and distilled, the distillation being continued until it no longer gives any color reaction with paradimethylamidobenzaldehyde. A portion of the distillate is now treated with a paradimethylamidobenzaldehyde solution made up by dissolving 15 grams of the aldehyde in 30 cc. of concentrated sulphuric acid and diluting this to 100 cc. The aldehyde solution thus prepared is added to the distillate until the maximum color reaction appears. This may be somewhat heightened by the addition of a small amount of concentrated hydrochloric acid. If indol is present in the distillate it should be removed by means of β -naphthaquinone-sodium-monosulphonate.¹ If phenol is present it must be got rid of by redistillation, it being held back by strong alkali. For clinical purposes the quantity of skatol present may be closely enough approximated by comparing the color obtained with Ehrlich's aldehyde with various dilutions of a watery solution of skatol of known strength. It is best to make the comparison after the contents of the test-tube have cooled, as this causes a deepening of the color toward blue.

I have made experiments with many kinds of bacteria (using pure cultures of bacteria and also using mixtures of bacteria) in the hope of learning something of the conditions under which skatol is formed rather than indol. Only a fair measure of success has been gained in this attempt. The main obstacle to success is the difficulty in obtaining a culture medium really comparable to that which the skatol-making bacteria find within the human intestine. If we inoculate ordinary culture media (peptone bouillon, plain agar, blood agar, milk) with mixed fecal

¹ A Method for the Quantitative Determination of Indol, this *Journal*, i, p. 257, 1906.

bacteria from a specimen containing skatol we are almost certainly disappointed in the hope that skatol will result in the course of the subsequent incubation. The putrefactive decomposition yields indol, not skatol. Only in rare instances is a trace of skatol found. From fluid media containing ground brain substance or fibrin one may obtain skatol, but the yields are seldom considerable and the putrefaction is usually indolic rather than skatolic. Sometimes after long putrefaction there is a considerable yield of skatol.

Difficulties have also been encountered in securing pure cultures of microorganisms capable of regularly giving rise to skatol when grown upon ordinary culture media. Marked irregularities in skatol formation have in some instances been observed where it has not been possible to accurately determine what conditions have been responsible for these irregularities. Nevertheless a few organisms out of a large number that were tried were found to produce skatol with considerable regularity when grown on peptone bouillon to which blood has been added. These organisms were a strain of malignant œdema obtained from Prof. Theobald Smith, two strains of *B. putrificus* and an unidentified putrefactive anaërobic organism sent me by Dr. Smith. It is noteworthy that the best success in obtaining skatol has come from the use of organisms that grow under anaërobic conditions. Some strains of *B. proteus* of Hauser are probably also to be regarded as skatol-producers. There are doubtless many other anaërobic bacteria, besides those mentioned, which are capable of forming skatol. I have not been able to satisfy myself that *B. aerogenes capsulatus* ever produces skatol, though some strains make indol. As to tetanus, I am also in doubt. I have never been able to obtain more than mere traces of skatol from any strain of colon bacillus in my possession, whether the organism was grown aerobically or anaërobically. Long cultivation was required to give even these insignificant traces.

It is certain that the conditions which lead to the formation of indol are fundamentally different from those leading to the production of skatol. The conversion of skatol into indol is one that might be thought of as possible for microorganisms to effect. I have made experiments with a view to determining whether colon bacilli which were energetic indol-formers could form indol

from skatol. The organisms were grown on a medium consisting of gelatin and salts to which a small quantity of skatol had been added. The organisms grew abundantly on this medium but even after many weeks' growth not a trace of indol could be detected. This result is in harmony with the observations made by Ellinger¹ on indol and skatol formation in the intestinal tract of the rabbit. He states that at most a mere trace of indol may possibly be formed from skatol. I question whether even this is likely. Indol has been produced from skatol by potash fusion but so severe and destructive a method as this cannot be compared with any powers which it is likely that bacteria can exert.

That both indol and skatol are derived from tryptophan cannot be doubted. One may easily satisfy oneself of the ability of tryptophan to yield skatol and indol by experimenting with media made up by the addition of tryptophan to gelatin, pure cultures of different bacteria being employed. There is no reason to suppose that any other constituent of the proteid molecule than tryptophan is able to yield indol and skatol. We have thus to look to the chemical constitution of tryptophan for a clue to the solution of the problem why skatol is sometimes formed and at other times indol. I cannot pretend to offer an adequate hypothesis upon this question, but would like to call attention to certain facts which point to the reasonableness of the idea that the formation of skatol in one case and the formation of indol in another, may really be conditioned by the nature of the intermediate products that arise before tryptophan can yield either one of these substances. It may now be regarded as settled that tryptophan is indol-amidopropionic acid and not skatol-amidoacetic acid, as was thought more likely by Hopkins and Cole. Moreover it is probable that tryptophan is an α -amido acid. It is certain that under the action of microorganisms tryptophan is capable of yielding indol-acetic acid, as was shown by Hopkins and Cole² to be true of a tryptophan medium inoculated with a pure culture

¹ Ueber die Constitution der Indol Gruppe im Eiweiss (Synthese der sogen. Skatolcarbonsäure) und die Quelle der Kynurensäure, *Ber. d. deutsch. chem. Gesellsch.*, no. 7, p. 1802, May 4, 1906.

² Called by them skatol carbonic acid. The Constitution of Tryptophane and the Action of Bacteria upon it. *Journ. of Physiol.*, xxix, p. 438, 1903.

of *B. coli communis*. This substance is probably not readily attacked by microorganisms. Some bacteria appear unable to attack indol-acetic acid. Thus *B. coli communis*, although able to form this substance from tryptophan either makes no skatol from it or only minute quantities. It is however probable that some bacteria (especially putrefactive anaerobes) are able to act upon indol-acetic acid in such a way as to cause it to lose carbon dioxide and such a change would explain the production of skatol. Assuming indol-acetic acid to be relatively unattackable by microorganisms, one would have an explanation of the usually small and slow yield of skatol in putrefaction.

Decomposition of tryptophan may, however, take another direction. Through oxidation, with the removal of the amido group, indol-propionic acid is formed. Assuming that this substance is relatively easily attacked by microorganisms, it is easy to see how indol and carbon dioxide might result from such bacterial attack, indol-carbonic acid being formed as an intermediate product. There is apparently no reason to suppose that indol-acetic acid is readily converted into indol-carbonic acid, since this calls for a process of energetic oxidation only likely to occur through the action of relatively powerful oxidizing agents. It would appear, then, that the formation of skatol may hinge on the antecedent production of indol-acetic acid, whereas the formation of indol may depend on the production of indol-propionic acid. To what extent this suggestion may be borne out by experimental facts can only be determined by further observations. Dr. Dakin has been so kind as to offer to prepare for me these important intermediary substances, the possession of which should serve to definitely determine their bacterial relation to indol and skatol.

The main conclusions which I desire to emphasize are the following:

1. Skatol is by no means always present in the contents of the lower gut in man. In healthy children it is only seldom detectable and then only in traces. In healthy adults it is frequently absent and when present occurs only in traces.
2. In some cases of excessive intestinal putrefaction skatol formation is considerably increased, often together with increased indol formation but sometimes without this.
3. There are instances in which the feces contain skatol but

no indol, despite the fact that the presence of indican in the urine points to indol formation in the intestine. As there is no evidence that indol is absorbed more rapidly than skatol in such cases, the presence of skatol without indol is probably due to the later production of the skatol.

4. Increased skatol production is observed in many persons suffering from excessive saccharo-butyric putrefaction due mainly to putrefactive anaërobic bacteria.

5. There are strains of the bacillus of malignant œdema and of *Bacillus putrificus* which form skatol. The *Bacillus coli communis* makes indol but usually no skatol or only mere traces.

6. The conditions giving rise to the formation of skatol are fundamentally different from those that govern the formation of indol. The formation of indol-acetic acid is perhaps a necessary step in the production of skatol, most bacteria attacking it with difficulty, if at all.

VII. ON SOME PICROLONATES: GUANIDINS.

By HENRY L. WHEELER AND GEORGE S. JAMIESON.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, December 13, 1907.)

In a paper recently published by one of us¹ on some salts of cytosin, isocytosin, 6-aminopyrimidin and 6-oxypyrimidin the melting or effervescing points of the picrolonates were given. When the these salts were first prepared it was found that Kjeldahl's method of analysis gave low results. The method was as follows: About 0.11-0.12 gram of substance was added to 20 cc. of sulphuric acid, then 2 or more grams of zinc dust and finally 15 grams of potassium sulphate; the digestion then being carried on as usual. On the other hand this method proved successful in the case of the corresponding picrates.

The picrolonic acid which we used was obtained from Kahlbaum. It was shown to be a pure sample by an absolute nitrogen determination and by a carbon and hydrogen determination, but the analysis of this material by Kjeldahl's method also gave results that were several per cent less than the calculated.

The difficulty was finally found to be due to the insolubility of picrolonic acid in concentrated sulphuric acid since Kjeldahl's procedure gave results agreeing with the calculated when the reduction was carried out in dilute sulphuric acid with the addition of some alcohol. Although numerous picrolonates have recently been described it appears that nitrogen in these salts is invariably been determined by the absolute method.²

Before correct results had been obtained in the analysis of our sample of picrolonic acid and in order to identify the material

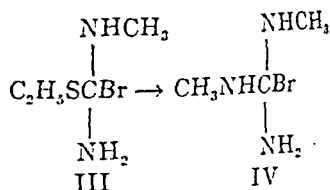
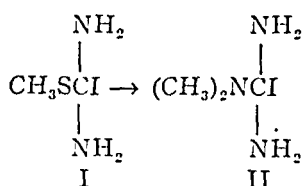
¹ This *Journal*, iii, p. 285, 1907.

² For a list of papers on picrolonates up to the year 1907, see Matthes and Rammstedt: *Archiv d. Pharmazie*, ccxlv, p. 112, 1907. Since then, Levene: *Biochem. Zeitschr.*, iv, p. 320, 1907, and Warren and Weiss: *This Journal*, iii, p. 327, 1907.

the guanidin salt was prepared. This salt agreed in properties with the description of guanidin picrolonate given by Schenck.¹

The action of aqueous solutions of amines, primary or secondary, or ammonia on the alkylhalide addition products of thioureas affords a very convenient method for the preparation of certain substituted guanidins, especially those that have been found in meat extract² and urine.³

The action takes place at ordinary temperature, mercaptan is liberated, and on evaporating, a guanidin salt is obtained. For example, when the methyl iodide addition product of thiourea, I (2-methylpseudothioureahydriodide), was dissolved in an excess of a 33 per cent solution of dimethylamine bubbles of methylmercaptan escaped. After evaporating off the excess of amine by warming, then adding a hot aqueous solution of picronic acid, unsymmetrical dimethyl guanidin (2, 2-dimethyl guanidin) picrolonate separated. This salt agreed with the description of dimethyl guanidin picrolonate given by Kutscher.⁴



The hydrobromide of the isomeric symmetrical dimethyl guanidin, IV (1, 2-dimethyl guanidin), was obtained by treating the ethylbromide addition product of methylthiourea, III, (1-methyl-2-ethylpseudothiourea) with methylamine.

The melting point of the picrolonate prepared from this substance was identical with that assigned to symmetrical dimethyl guanidin picrolonate by Schenck.⁵

Monomethyl guanidin hydrobromide was prepared in two ways, by dissolving the ethylbromide addition product of thiourea in an aqueous solution of methylamine and by dissolving the corresponding addition product of methylthiourea in concentrated

¹ *Zeitschr. f. physiol. Chem.*, xlv, p. 427, 1905.

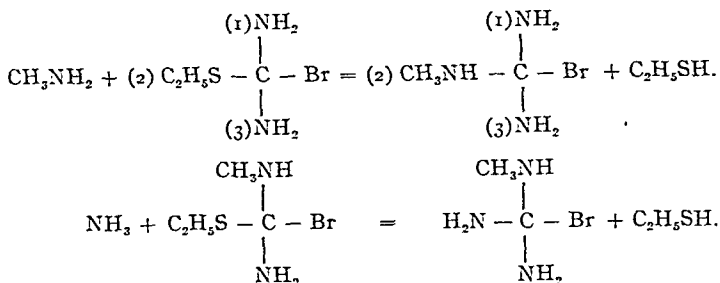
² Kutscher and Lohmann: *Zeitschr. f. physiol. Chem.*, xlviii, p. 1, 1906.

³ Achelis: *Ibid.*, l, p. 10, 1906; Kutscher: *Ibid.*, li, p. 457, 1907.

⁴ *Loc. cit.*

⁵ *Zeitschr. f. physiol. Chem.*, xlviii, p. 423, 1906.

ammonia. As would be expected from the non-existence of tautomeric forms in the case of the amidines¹ the picrolonates of the material prepared by these two methods were identical. The reactions and the substances may be represented as follows:



The nomenclature hitherto employed for pseudothiureas and guanidins (symmetrical and unsymmetrical) is not sufficient to define substituted derivatives in general. The writer therefore proposes to use that system which has been found so convenient in the purin² and pyrimidin series, namely, of assigning numbers (as shown above) to indicate the position of substituents in the case of the pseudoarea, pseudothiurea and guanidin derivatives. Dieckmann³ has used this system for parabanic acid compounds.

This then brings about a systematic nomenclature for all these related substances.

EXPERIMENTAL PART.

Cytosin Picrolonate, $\text{C}_4\text{H}_5\text{ON}_3 \cdot \text{C}_{10}\text{H}_9\text{O}_5\text{N}_4$.—A half a gram of cytosin in a 100 cc. of water was added to one gram of picrolonic acid in about 350 cc. of hot water. On rapidly cooling the solution an amorphous, gelatinous precipitate separated. On slowly cooling or on crystallizing from water little balls of fine bright yellow needles or slender prisms separated. This salt is very difficultly soluble in alcohol from which it is obtained in flat prisms or plates. It melts with effervescence about 270° – 273° .

The nitrogen determinations in the case of this and the other picrolonates were determined by Kjeldahl's method as follows:

¹ Wheeler and Johnson: *Amer. Chem. Journ.*, xxxi, p. 577, 1904.

² *Ber. d. deutsch. chem. Gesellsch.*, xxx, p. 549, 1897.

³ *Ibid.*, xl, p. 3737, 1907.

About 0.1 gram of substance was dissolved in a mixture of 35 cc. of alcohol and 20 cc. of dilute sulphuric acid. Two grams of zinc dust were used for the reduction and the solution was warmed until colorless. Then 10 cc. of concentrated sulphuric acid and 12 grams of potassium sulphate were added, the water and alcohol was evaporated and the residue was digested for two or three hours. The analysis was then finished in the usual manner.

	Calculated for $C_{14}H_{13}O_6N_7$:	Found:
N	26.13	26.15

Since this salt was prepared by us it has been obtained by Levene.¹ He gives the formula $C_4H_6N_3 \cdot C_{10}H_8O_5N_4$, which is evidently a typographical error.

2-Amino-6-oxypyrimidin (Isocytosin) Picrolonate, $C_4H_5ON_3 \cdot C_{10}H_8O_5N_4$.—A half a gram of isocytosin and 1.2 gram of picrolonic acid, on mixing the hot aqueous solutions (about 400 cc.), gave an immediate, bulky precipitate consisting of very fine, yellow hair-like needles. This salt is practically insoluble in alcohol and its melting or effervescing point varies with the rate of heating. It usually decomposes about 273° - 275° .

	Calculated for $C_{14}H_{13}O_6N_7$:	Found:
N	26.13	26.05

6-Aminopyrimidin Picrolonate, $C_4H_5N_3 \cdot C_{10}H_8O_5N_4$.—A solution of 0.1 gram of 6-aminopyrimidin in 4 cc. of water was added to 0.3 gram picrolonic acid in 100 cc. of hot water. This gave an immediate precipitate and on cooling, the salt separated in the form of interlaced, yellow, hairlike needles. The yield was 0.35 gram, calculated, 0.4 gram. It melted with effervescence at 261° ; on rapidly heating it melted a few degrees higher.

	Calculated for $C_{14}H_{13}O_6N_7$:	Found:
N	27.29	27.10

6-Oxypyrimidin Picrolonate, $C_4H_4ON_2 \cdot C_{10}H_8O_5N_4$.—A solution of 0.2 gram of 6-oxypyrimidin in 6 cc. of water was added to a hot solution of 0.55 gram picrolonic acid in 125 cc. of water.

¹ *Biochem. Zeitschr.*, iv, p. 320, 1907.

This gave an immediate precipitate and on cooling, a bulky mass of slender, yellow needles separated. The yield was 0.55 gram. When this was heated it showed signs of change at 180° and then melted with vigorous effervescence at 222° . The salt was recrystallized from water and from alcohol. It then melted suddenly, in both cases, at 191° – 193° . It is difficultly soluble in alcohol and it separates from this solvent in thin scales or plates.

	Calculated for $C_{14}H_{12}O_6N_6$	Found:
N	23.33	23.33

Methylguanidin Picrolonate, $C_2H_7N_3 \cdot C_{10}H_8O_5N_4$.—2-Ethyl-pseudothiourea hydrobromide was dissolved in an excess of a 33 per cent solution of methylamine and allowed to stand over night. The excess of amine and mercaptan was then evaporated and the solution, after filtering from a slight turbidity, was divided into two parts. One part was precipitated with picric acid; the picrate formed needles which melted sharply at 200° . Fischer¹ gives the melting point of methyl guanidin picrate at 200° .

The remaining solution was precipitated with an aqueous solution of picrolonic acid. The salt formed a compact, not bulky, yellow precipitate which when recrystallized from water formed minute, diamond shaped tables or blocks. It decomposed with effervescence at 291° .

	Calculated for $C_{12}H_{15}O_5N_7$	Found:
N	28.24	28.23

1-Methylguanidin was also prepared by dissolving 1.5 gram of 1-methyl-2-ethylpseudothiourea hydrobromide in concentrated ammonia. On evaporating the solution and adding an aqueous solution of picrolonic acid the precipitate obtained was identical with the above.

2,2-Dimethylguanidin Picrate, $C_3H_9N_3 \cdot C_6H_3O_7N_3$.—The guanidin was prepared by dissolving 2-ethylpseudothiourea hydrobromide in a strong, aqueous solution of dimethylamine. The precipitate produced by adding an aqueous solution of picric acid was crystallized from water. It formed small, pointed,

¹ Ber. d. deutsch. chem. Gesellsch. xxx, p. 2414, 1897.

About 0.1 gram of substance was dissolved in a mixture of 35 cc. of alcohol and 20 cc. of dilute sulphuric acid. Two grams of zinc dust were used for the reduction and the solution was warmed until colorless. Then 10 cc. of concentrated sulphuric acid and 12 grams of potassium sulphate were added, the water and alcohol was evaporated and the residue was digested for two or three hours. The analysis was then finished in the usual manner.

	Calculated for $C_{14}H_{13}O_6N_7$:	Found:
N	26.13	26.15

Since this salt was prepared by us it has been obtained by Levene.¹ He gives the formula $C_4H_6N_3 \cdot C_{10}H_8N_4O_5$, which is evidently a typographical error.

2-Amino-6-oxypyrimidin (Isocytosin) Picrolonate, $C_4H_5ON_3 \cdot C_{10}H_8O_5N_4$.—A half a gram of isocytosin and 1.2 gram of picrolonic acid, on mixing the hot aqueous solutions (about 400 cc.), gave an immediate, bulky precipitate consisting of very fine, yellow hair-like needles. This salt is practically insoluble in alcohol and its melting or effervescing point varies with the rate of heating. It usually decomposes about 273° – 275° .

	Calculated for $C_{14}H_{13}O_6N_7$:	Found:
N	26.13	26.05

6-Aminopyrimidin Picrolonate, $C_4H_5N_3 \cdot C_{10}H_8O_5N_4$.—A solution of 0.1 gram of 6-aminopyrimidin in 4 cc. of water was added to 0.3 gram picrolonic acid in 100 cc. of hot water. This gave an immediate precipitate and on cooling, the salt separated in the form of interlaced, yellow, hairlike needles. The yield was 0.35 gram, calculated, 0.4 gram. It melted with effervescence at 261° ; on rapidly heating it melted a few degrees higher.

	Calculated for $C_{14}H_{13}O_5N_7$:	Found:
N	27.29	27.10

6-Oxypyrimidin Picrolonate, $C_4H_4ON_2 \cdot C_{10}H_8O_5N_4$.—A solution of 0.2 gram of 6-oxypyrimidin in 6 cc. of water was added to a hot solution of 0.55 gram picrolonic acid in 125 cc. of water.

¹ *Biochem. Zeitschr.*, iv, p. 320, 1907.

This gave an immediate precipitate and on cooling, a bulky mass of slender, yellow needles separated. The yield was 0.55 gram. When this was heated it showed signs of change at 180° and then melted with vigorous effervescence at 222° . The salt was recrystallized from water and from alcohol. It then melted suddenly, in both cases, at 191° – 193° . It is difficultly soluble in alcohol and it separates from this solvent in thin scales or plates.

	Calculated for $C_{14}H_{12}O_6N_6$	Found:
N	23.33	23.33

Methylguanidin Picrolonate, $C_2H_7N_3 \cdot C_{10}H_8O_5N_4$.—2-Ethyl-pseudothiourea hydrobromide was dissolved in an excess of a 33 per cent solution of methylamine and allowed to stand over night. The excess of amine and mercaptan was then evaporated and the solution, after filtering from a slight turbidity, was divided into two parts. One part was precipitated with picric acid; the picrate formed needles which melted sharply at 200° . Fischer¹ gives the melting point of methyl guanidin picrate at 200° .

The remaining solution was precipitated with an aqueous solution of picrolonic acid. The salt formed a compact, not bulky, yellow precipitate which when recrystallized from water formed minute, diamond shaped tables or blocks. It decomposed with effervescence at 291° .

	Calculated for $C_{12}H_{15}O_5N_7$	Found:
N	28.24	28.23

1-Methylguanidin was also prepared by dissolving 1.5 gram of 1-methyl-2-ethylpseudothiourea hydrobromide in concentrated ammonia. On evaporating the solution and adding an aqueous solution of picrolonic acid the precipitate obtained was identical with the above.

2,2-Dimethylguanidin Picrate, $C_3H_9N_3 \cdot C_6H_3O_7N_3$.—The guanidin was prepared by dissolving 2-ethylpseudothiourea hydrobromide in a strong, aqueous solution of dimethylamine. The precipitate produced by adding an aqueous solution of picric acid was crystallized from water. It formed small, pointed,

¹ Ber. d. deutsch. chem. Gesellsch. xxx, p. 2414, 1897.

yellow prisms or branch-like growths. It melted to an oil without effervescence at 224° .

	Calculated for $C_6H_{12}O_7N_6$:	Found:
N	26.58	26.53

2,2-Dimethylguanidin Picrolonate, $C_3H_6N_3 \cdot C_{10}H_8O_5N_4$.—The guanidin was prepared as above and also by the action of dimethylamine on 2-methylpseudothiourea hydriodide. The solutions gave an immediate precipitate with aqueous picrolonic acid. The salt was crystallized from water, it then formed small, flat, four-sided, yellow prisms which decomposed with effervescence quite sharply at 278° . Kutscher and Lohmann give the melting point of their dimethylguanidin picrolonate obtained from urine at 275° – 278° .¹

This salt is very difficultly soluble in alcohol from which it forms needle-like prisms.

	Calculated for $C_{13}H_{17}O_8N_7$:	Found:
N	27.13	27.13

1,2-Dimethylguanidin Picrate, $C_3H_6N_3 \cdot C_6H_3O_7N_3$.—One gram of 1-methyl-2-ethylpseudothiourea hydrobromide was treated with an excess of strong aqueous methylamine solution. The picrate formed yellow prisms with uneven striated faces. It melted to a clear oil at 178° .

	Calculated for $C_9H_{12}O_7N_6$:	Found:
N	26.58	26.58

A portion of the guanidin solution was precipitated with picrolonic acid. This precipitate crystallized from water in thin, yellow plates which melted at 262° . It agreed with the description given by Schenck² who gives the melting point 260° – 262° . This also confirms the conclusion of Kutscher and Lohmann that the dimethyl guanidin from urine has the isomeric structure.

In preparing guanidins by the above method an excess of amine must be used and the solutions should be strong, in order to bring about a complete reaction. Otherwise unaltered pseudothiourea will be precipitated as picrate or picrolonate.

¹ *Zeitschr. f. physiol. Chem.*, xlviii, p. 423, 1906.

² *Ibid.*

2-Ethylpseudothiourea Picrate, $C_3H_8N_2S.C_6H_3O_7N_3$, separates from aqueous solutions in flat, yellow prisms which melt at 184° to a clear oil.

	Calculated for $C_9H_{11}O_7N_5S$:	Found:
N	21.02	21.00

2-Ethylpseudothiourea Picrolonate, $C_3H_8N_2S.C_{10}H_8O_5N_4$, forms difficultly soluble, flat, yellow prisms which melt at 225° .

	Calculated for $C_{13}H_{16}O_5N_6S$:	Found:
N	22.82	22.69

1-Methyl-2-ethylpseudothiourea forms a picrate which crystallizes in yellow prisms and melts to a clear oil at 157° .

ESTIMATIONS OF ARGININ, LYSIN AND HISTIDIN IN PRODUCTS OF HYDROLYSIS OF VARIOUS ANIMAL TISSUES.

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(Received for publication, January 13, 1908.)

- I. Introduction.
- II. Normal tissues of various animals.
- III. Liver of the horse.
- IV. Liver of the dog.
- V. Kidney of the dog.
- VI. Muscle of the dog.
- VII. Liver of man.
- VIII. Summary.

I. INTRODUCTION.

In a previous investigation¹ on protein material from the liver tissue of dogs some results were reported bearing on the amounts of certain of its cleavage products obtained by acid hydrolysis, as well as the mutual relation of the products among themselves. The object of the investigation was to throw, if possible, some additional light upon the nature and changes of the protein substance under varying conditions. Normal and degenerated tissues were studied and the mutual quantitative relations of the cleavage products, the bases, or diamino acids, arginin and lysin, and the base histidin, were determined and the relation of their nitrogen content to the total nitrogen established. As a result of the study it was found that the proteins of cells with advanced degeneration resulting from severe phosphorus poisoning, when compared with the normal, were undergoing chemical changes

¹ "On the Hexon Bases of Liver Tissues under Normal and Certain Pathological Conditions." *Journ. of Exper. Med.*, vii, p. 292, 1905.

tending toward a diminution of those portions of the protein molecule which yield bases on hydrolysis.¹

It seemed well to extend the investigations along somewhat similar lines, not only upon liver tissue of dogs in varying pathological conditions, but upon other tissues of the dog, as the kidney and muscle, as well as upon the organs of other animals.

The present investigation includes a series of twenty-one analyses of the liver of the horse and dog, the kidney and muscle of the dog, and on the liver of man, as shown in more detail below.

Horse, liver, 2 normal, Table II.	Man, liver, 2 normal, Table VI.
" " 1 immune	" " 1 cirrhosis.
Dog, " 1 ricin, Table III.	" " 1 acute yellow atrophy,
" " 1 phloridzin.	" " 1 acute streptococcus septicæmia.
" " 1 fasting.	" " 1 puerperal eclampsia.
" kidney, 1 normal, Table IV.	" " 2 yellow fever.
" " 1 blood supply shut off.	" " 1 miliary tuberculosis of lungs.
" muscle, 2 normal Table V.	" " 1 infant.
" " 1 degenerated.	

The method employed throughout this investigation was the same as that outlined in the previous paper.²

From the finely divided tissue, two small portions were taken in each case for the estimation of total solids by drying to a constant weight at 105° C.; on two other small portions nitrogen estimations were made, and controlled by determining the nitrogen on the dried portions. These latter results were found as a rule to be just a shade lower than the former, and in the tables which follow the total nitrogen is based on the determination of the nitrogen in the moist tissue.³

In each case hydrolysis was accomplished by boiling one part of the moist tissue with three parts of concentrated sulphuric acid, and six parts of water for fourteen hours. One hundred and

Average from four normal dogs' livers:			Average from two phosphorized dogs' livers:		
Total nitrogen	=	100.	Total nitrogen	=	100.
Nitrogen of arginin	=	9.32	Nitrogen of arginin	=	4.59
" " lysin	=	4.76	" " lysin	=	3.19
" " histidin	=	2.29	" " histidin	=	1.53
		<hr/> 16.37			<hr/> 9.31

² *Loc. cit.*

³ Nitrogen estimations throughout the whole investigation were made in pairs by the Kjeldahl method.

fifty grams of the tissue were usually taken for analysis. The general method employed for the separation of the bases was that of Kossel and Kutscher, with some modifications. In the final fluids, after the separation of the bases, the content of the arginin, lysin and histidin was found by making nitrogen determinations, controlled in a few instances by crystallizing and weighing the final products as arginin nitrate, lysin picrate and histidin dichloride. The results reported are uniformly based on the nitrogen determinations. In the tables which follow, the nitrogen of the bases is expressed in three different ways: in percentage of the total solids, which indicates their absolute amounts; in percentage of the total nitrogen, indicating their proportion in the protein substance; and finally in percentage of the total nitrogen of the three bases, which indicates their mutual relationship.

II. NORMAL TISSUES OF VARIOUS ANIMALS.

Before presenting the results obtained from the study of pathological organs, it seems desirable to present the results thus far obtained from various normal organs of different animal species. For ease of comparison such results are brought together in Table I, on p. 123.

In viewing the results obtained from the liver of man, dog, horse, and fish, one is impressed with the degree of uniformity which exists, indicating that the difference of animal species influences but slightly the composition of the liver protein. The most extreme results are from the liver of the fish. It is seen here that the nitrogen of the three bases is but 12.59 per cent of the total nitrogen, while that of the other three animals varies from 15.49 per cent to 13.12 per cent, the average being 14.69 per cent. The interpretation to be placed upon these results is that in the liver of the fish that portion of the protein substance not yielding the bases is relatively richer in nitrogen than is the case in regard to the other animals under consideration. In drawing distinctions of this kind, however, there are certain influencing factors which it is necessary to keep in mind. It is undoubtedly true, in the first place, that in the same organ of a given animal species there may be a considerable normal varia-

tion, not only as regards the mutual relation of the atomic groups of the protein molecules from which the bases are derived, but also of these groups as a whole to the entire molecule. Illustrating this latter point, the livers from two normal adults showed, in the one case, nitrogen of the bases equivalent to 12.9 per cent of the total nitrogen; in the other, 18.1 per cent, a wide variation. In the case of the four normal dogs studied, the lowest value for nitrogen of the bases was 12.6 per cent of the total nitrogen; the highest was 18.1 per cent. In the second place it must be borne in mind, while interpreting such results as the nitrogen of the bases in percentage of the total nitrogen, that the total nitrogen not only includes all the nitrogen of the protein molecule, but the nitrogen of various other bodies, such as lecithin, jecorin, the coloring matter of blood and bile, and urea. The amount of this extraneous nitrogen, if we may so term it, is probably so small compared with the nitrogen derived from the protein substance, entering as it does into all values based on total nitrogen as a common error, within necessarily narrow limits of variation, that its influence in the interpretation of the results can be but slight, and in the comparison of values to which it is common, its influence is certainly negligible. Finally in the interpretation of the results one must bear in mind the nature of the chemical procedure, which is involved and subject to limitations.

While, therefore, the nitrogen of the bases in the case of the fish seems low compared with other animals, showing a relative preponderance of nitrogen in other portions of the protein molecule than that related to the bases, this difference is subject normally to the influence of various outside factors. The relation of the bases to one another, in the case of the fish, is seen to bear close resemblance to that observed from the liver proteins of the higher animals. This relationship is seen in the last three columns of the table, where the nitrogen of the bases is expressed in percentage of the total nitrogen of the three bases. In viewing these results one is impressed with the superior constancy of the histidin value, indicating that the other two groups in the protein molecule of the normal tissue, from which arginin and lysin are derived, are the more labile. One would certainly expect a greater similarity of behavior between those atomic groups

TABLE I.

NATURE OF EXPERIMENT.		Case.	DRY substance in 100 parts of moist tissue.		DRY SUBSTANCE = 100.				TOTAL NITROGEN OF DRY SUBSTANCE = 100.				TOTAL NITROGEN OF BASES = 100.			
LIVER, MUSCLE, KIDNEY.			Total nitrogen.	NITROGEN OF BASES.			NITROGEN OF BASES.				NITROGEN OF BASES.					
No.				Arginin.	Lysin.	Histidin.	The three bases.	Arginin.	Lysin.	Histidin.	Arginin and Lysin.	The three bases.	Arginin.	Lysin.	Histidin.	
I	Man.	Normal liver	25.08	11.89	0.819	0.785	0.239	1.84	6.88	6.60	2.01	13.48	15.49	44.4	42.6	13.0
II	"	"	27.19	11.73	0.972	0.561	0.272	1.81	8.32	4.81	2.31	13.13	15.44	53.9	31.2	14.9
III	Horse.	"	29.48	11.23	0.642	0.633	0.197	1.47	5.72	5.64	1.76	11.36	13.12	43.6	43.0	13.4
Av.	I, II, III.	27.24	11.62	0.811	0.660	0.236	1.72	6.97	5.69	2.03	12.66	14.69	47.3	38.9	13.8
IV	Fish (sturgeon) liver.	28.84	7.07	0.479	0.286	0.124	0.89	6.78	4.05	1.76	10.83	12.59	53.8	32.2	14.0
V	Dog.	Normal muscle.	28.28	12.50	1.197	1.053	0.368	2.62	9.46	8.44	2.94	17.90	20.84	45.4	40.5	14.1
VI	Dog.	" kidney	21.43	12.17	0.511	0.737	0.306	1.55	4.19	6.05	2.51	10.24	12.75	32.9	47.4	19.7

I. Average of two series of results from two adult normal livers (Table VI).

II. Average of four series of normal results, three having been previously reported (*loc. cit.*). One animal had received 0.1 gm. of potassium cyanide by subcutaneous injections in small doses distributed over a few hours immediately preceding death in a blood pressure experiment. The animal was killed by bleeding. Histological examination of the liver showed it to be perfectly normal.

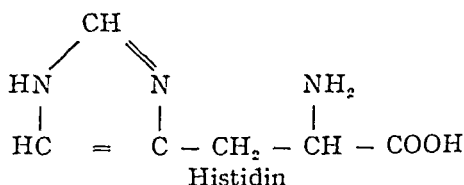
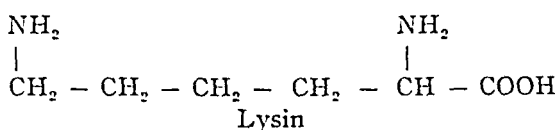
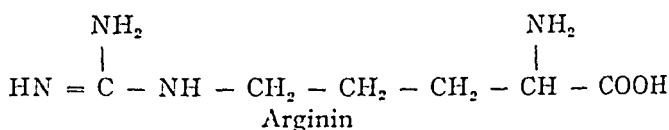
III. One series (Table II).

IV. One series reported in the *Zeitschrift für physiologische Chemie*, xiv, p. 341, 1905.

V. Average of two series of results from muscles of two normal dogs (Table V).

VI. Results obtained from a mixture of four kidneys from four normal dogs (Table IV).

from which the arginin and lysin are derived, than between either of these and the group yielding histidin, on the ground of the chemical structure of the bases. Arginin and lysin are much more nearly related to one another in chemical structure than is arginin to histidin or lysin to histidin, as seen by the following formulæ:



The histidin molecule, by nature of its cyclic structure stands quite apart, so one may question if there be any close relation in the protein molecule between the atomic groups from which the histidin is derived and the other groups yielding arginin and lysin.

The nitrogen of bases from the protein of normal dog's muscle is seen to be 20.84 per cent of the total nitrogen.¹ This is strikingly higher than the corresponding average value from the livers of man, dog and horse, which is 14.69 per cent. If this difference is not due to the influence of outside factors (and the effect of such factors is here minimized since the values compared are averages), it indicates a considerable difference in the molecular structure of the liver and muscle proteins, the latter being richer in the atomic groups from which the bases originate. The relation of the bases to one another is seen to be very similar to that in the liver protein.

¹ The individual values obtained from the muscle from two normal dogs were identical (Table V).

The results obtained from normal kidney protein are from a single analysis, and are not as representative, therefore, as if averaged from several series, although the material on which the bases were estimated was from a mixture of kidney tissue from four normal dogs. These results show a greater divergence than is found in any of the other observations. The nitrogen of the bases is low, being but 12.75 per cent of the total nitrogen; but even a greater variation is seen in the relation of the bases to one another, the arginin being particularly low.

III. LIVER OF THE HORSE.

In order to test the question whether changes occur in the liver protein sufficiently profound to be detected by the method at hand, in an animal which had been rendered immune by a long course of treatment with a toxic serum, application was made to Dr. William H. Park, of the Department of Health, New York, for the liver of a horse immune to diphtheria toxin. Through his kindness such a liver was obtained. It was deemed possible that the action of diphtheria toxin in stimulating the living cell elements to the overproduction of those substances which constitute antitoxin, might perhaps be attended by alterations in the composition of the tissue proteins.

In the following Table II, p. 126, the results from the analysis of the liver of the immune horse are recorded, and compared with results obtained from the liver of a normal horse. For the histological examinations reported here and elsewhere in the paper I am indebted to Dr. William R. Williams of Columbia University.

On the normal specimen two analyses were carried through, side by side, in order to be able to arrive at some conclusion as to the probable discrepancy due to the method. The agreement between the arginin and lysin values is surprisingly close, but considerable variation exists between the histidin values.

In comparing the results obtained from the livers of the immune and normal animals, an increase in the nitrogen of the bases is observed in the case of the liver of the immune, amounting to over 2.5 per cent of the total nitrogen. This increase is noticeable in the case of the arginin and histidin, the lysin nitrogen content being much alike in the two livers.

TABLE II.

NATURE OF EXPERIMENT.		Dry substance in 100 parts of moist tissue.	DRY SUBSTANCE = 100.				TOTAL NITROGEN OF DRY SUBSTANCE = 100.					TOTAL NITROGEN OF BASES = 100.				
Horse.	LIVER.		Case.	NITROGEN OF BASES.			NITROGEN OF BASES.					NITROGEN OF BASES.				
				Total nitrogen.	Arginin.	Lysin.	Histidin.	The three bases.	Arginin.	Lysin.	Histidin.	Arginin and Lysin.	The three bases.	Arginin.	Lysin.	Histidin.
I	Normal.....	{	29.48	11.23	0.642	0.633	0.197	1.47	5.72	5.63	1.76	11.35	13.11	43.6	43.0	13.4
II	Immune.....	{	29.48	11.23	0.643	0.647	0.137	1.43	5.73	5.77	1.23	11.49	12.73	45.1	45.4	9.5
			28.89	11.85	0.882	0.704	0.287	1.87	7.44	5.94	2.42	13.38	15.80	47.1	47.1	137.6

I. Normal horse. The animal was killed by bleeding, and the liver tissue was brought into contact with strong sulphuric acid without delay. Histological examination showed the liver to be normal.

II. Horse immune to diphtheria toxin. The animal had been under treatment about eight months and shortly before he was killed the serum test was 300 units per cubic centimeter of serum; at the time of death it was 200. The horse had received five days before death, 1000 cc. of diphtheria toxin, 1 cc. killing 200 guinea pigs. The animal was killed by bleeding and the liver reached the laboratory without delay. Histological examination of the liver showed slight fatty degeneration.

It is a question whether these differences can be connected in any way with the peculiar condition of immunity. There is some evidence to the contrary, as seen in the results from the liver of a normal dog and the liver of a dog immune to ricin, shown in a later table. Here the results are quite the opposite. In both cases, however, the values probably fall within the limits of normal variation. As far as this experiment goes, therefore, there is no indication that the composition of the protein substance is affected.¹

IV. LIVER OF THE DOG.

It was thought desirable to learn whether the liver undergoes in phlorhizin poisoning any changes comparable with those that occur from the action of phosphorus. An observation was also made on the effects of ricin since it is known that this substance is capable of inducing profound histological changes in the liver, differing from those found either in phlorhizin or phosphorus poisoning. As starvation is a feature in all these forms of poisoning it was clearly necessary to see whether mere fasting is capable of inducing changes in the liver protoplasm. The results² are recorded in Table III, p. 128.

¹ Jackson and Pearce (Liver Necrosis, *Journ. of Exper. Med.*, ix, p. 520, 1907) found considerably lower values in the analyses of two normal horse livers. The nitrogen of the three bases they found to amount to 7.6 per cent and 7.0 per cent of the total nitrogen. The nitrogen of arginin plus histidin was found to be 3.0 per cent and 3.9 per cent of the total nitrogen, the lysin nitrogen being 4.6 per cent and 3.1 per cent. Of the nitrogen of the bases, arginin and histidin together yielded 39.5 per cent, lysin 60.5 per cent in the one case; in the other, arginin and histidin yielded 55.7 per cent and lysin 44.3 per cent.

² It appears from analyses that the bases obtained from different normal organs of the same kind are subject to considerable variation. In normal dog's muscle, for instance, the nitrogen of the bases from one was 20.86 per cent of the total nitrogen, and from the other 20.83 per cent, while the nitrogen of arginin in one case was 10.32 per cent and in the other, 8.61 per cent of the total nitrogen (Table V). In the two normal human livers examined the nitrogen of the bases in one case was 12.91 per cent of the total nitrogen, in the other, 18.08 per cent. The nitrogen of arginin in the former was 5.61 per cent, and in the latter, 8.16 per cent of the total nitrogen (Table VI). The extreme values among the four normal dogs gave for nitrogen of bases 18.1 per cent and 12.6 per cent of total nitrogen,

TABLE III.

Dog.	NATURE OF EXPERIMENT.	LIVER.	Case.	Dry substance in 100 parts of moist tissue.	DRY SUBSTANCE = 100.				TOTAL NITROGEN OF DRY SUBSTANCE = 100.				TOTAL NITROGEN OF BASES = 100.			
					Total nitrogen.	NITROGEN OF BASES.			Arginin.	Lysin.	Histidin.	Arginin and Lysin.	The three bases.	Arginin.	Lysin.	Histidin.
						Arginin.	Lysin.	Histidin.								
I } II } III } IV }																
V			Normal.....	27.19	11.73	0.97	0.56	1.81	8.32	4.81	2.31	13.13	15.44	53.9	31.2	14.9
VI			Ricin.....	28.99	12.31	0.81	0.71	0.24	1.77	6.63	5.83	1.98	12.46	14.44	45.9	40.4
VII			Phlorizin.....	36.66	9.62	0.32	0.60	0.58	0.14	1.05	4.17	7.42	1.80	11.59	13.39	31.1
AV.			Fasting.....	27.97	12.81	0.67	0.74	0.22	1.65	5.27	5.80	1.79	11.07	12.86	41.0	45.1
			V, VI, VII.....													
									5.36	6.35	1.86	11.71	13.57	39.5	46.8	13.7

I, II, III, IV. Normal animals.

V. Ricin. Dog. Weight, 17 kilos.

Oct. 1. Subcutaneous injection 0.5 mg. ricin in dilute solution. Animal much prostrated.

Oct. 5. 0.5 mg. ricin again injected, followed by symptoms, but less severe.

Oct. 10. 0.5 mg. ricin injected. Animal recovering.

Oct. 14. 0.5 mg. ricin injected. Slight symptoms.

Oct. 16. 0.5 mg. ricin injected. No symptoms.

Oct. 17-20 inclusive. 0.5 mg. ricin injected daily. No symptoms.

Oct. 21-23 inclusive. 0.6 mg. ricin injected daily. No effects.

Oct. 24. 0.9 mg. ricin injected. No effects.

Oct. 25. The animal was killed by opening the femoral artery. Histological examination of liver: fatty degeneration near center of lobule to moderate degree. Nuclei of all cells do not stain. Capillaries full of blood.

VI. Phlorizin dog. Weight, 13 kilos.

The second and third day following he received 2 gms. of phlorizin daily, by subcutaneous injection in moderate solution, for six consecutive days.

The animal showed much emaciation. He was killed by bleeding. The liver was exceedingly fatty, showing the typical appearance of phlorizin poisoning. Many nuclei do not stain.

VII. Fasting dog. Weight, 5.64 kilos.

After thirteen days of fasting the animal weighed 4.81 kilos; sixteen days later the dog weighed 3.36 kilos having lost 40 per cent. of his original weight.

At the end of this period, a total of 29 days, the dog was exceedingly emaciated, and was killed by bleeding. Weight of liver, 107 grams.

The liver showed the presence of acetone and the absence of diacetic acid. By histological examination, the tissue appeared perfectly normal.

In viewing the results given in the above table it is seen that the sum of the three bases varies within fairly narrow limits. The average of the nitrogen of the three bases in the case of the normal animals is found to be 15.96 per cent of the total nitrogen. The average percentage of nitrogen of the three bases in the case of the three pathological dogs is 13.57, between the limits 14.44 and 12.86. These values from the normal as well as the pathological livers are considerably higher than those reported by Jackson and Pearce.¹

In connection with the fairly constant total nitrogen values for the three bases from the normal and pathological livers, it is interesting to note the strikingly constant values for the nitrogen of histidin. The normal average percentage is 2.31 of the total nitrogen; the average from the three pathological dogs is 1.86, between the limits 1.98 and 1.79. This similarity of results among the total nitrogen of the bases as well as the nitrogen of the histidin is well brought out in the last column of the table. Here it is seen that the percentage of nitrogen of histidin, to the total nitrogen of the bases, as the average of normal values, is 14.9 per cent; the average from the pathological animals is 13.7 per cent, between the limits 13.9 and 13.5. This would seem to indicate that even if a change of balance is effected between that portion of the protein molecule from which the bases spring and the whole molecule, the balance between that part of the mole-

the values of nitrogen of arginin being 10.2 per cent and 5.4 per cent, respectively. These instances illustrate to some degree the extent of normal variation, but in the light of the various results obtained upon normal organs as expressed in Table I, and from comparisons between the results from normal and pathological human livers expressed in Table VI, it is probable that the arginin value of nitrogen from normal livers expressed in the above table is toward the upper limit of normal variation, and the lysin toward the lower. If this is so, the significance of the variation between the arginin nitrogen values obtained from the normal and pathological livers, as expressed in the table, becomes less; the same is true in the case of lysin.

¹ *Loc. cit.* Jackson and Pearce found the nitrogen of the bases in the three normal dogs' livers to be 7.9 per cent, 10.9 per cent and 14.5 per cent of the total nitrogen, average = 11.1 per cent. The nitrogen of arginin + histidin in the three cases was found to be 4.3 per cent, 6.0 per cent and 7.1 per cent of the total nitrogen, the nitrogen of lysin being 3.6 per cent, 4.9 per cent and 7.4 per cent of the total nitrogen.

cule from which the histidin springs and that larger part from which the three bases originate runs parallel.

In viewing these results considerable mutual variation is seen to exist among the values for arginin nitrogen and lysin nitrogen. The normal percentage of arginin nitrogen to the total nitrogen is 8.32; the average from the three pathological dogs is 5.36, between the limits 6.63 and 4.17. With lysin, the variation is similar, but in the opposite direction. The normal is 4.81; the average from the pathological animals is 6.35, between the limits 5.80 and 7.42. This mutual variation between the arginin and lysin nitrogen is more clearly shown in the last columns but one of the table. This relation can scarcely be explained on the ground of difficulties inherent in the method of analysis.¹

It might be suggested that in the pathological cases conditions may be more favorable for autolysis, and the atomic group from which histidin springs, being probably the most resistant by virtue of its molecular structure, the effect of any autolytic action might be expected to first show itself among the other groups. If some autolysis takes place through the action of the ferment arginase, then one would expect that portion of the molecule from which arginin is derived to be partially broken down with the formation of ornithin. Ornithin, it is remembered, is very similar to lysin in its chemical behavior; in fact, it is almost impossible to separate the two. Ornithin would therefore be estimated with the lysin. This explanation, quite hypothetical it is true, would account for the mutual relation appearing to exist between the arginin and the lysin. On this explanation, the values for lysin, being based on nitrogen estimation, would not necessarily be the true values, but would tend to be high as the arginin becomes low. It may be that the group from which lysin springs is, like histidin, relatively stable (as it would appear) and its variations as shown in the table only apparent.

¹ It might be noted that the separation of lysin from arginin as well as from histidin is unquestionable, on account of the decided difference of behavior of the bodies toward the reagents employed. The separation of histidin from arginin, on the other hand, is not so sharp; some arginin may be thrown out with the histidin by too abundant use of the reagent, or some histidin may be carried along with the arginin if an insufficient amount of the reagent is used.

Comparing the individual values from the pathological animals with the normals, it is seen that the nitrogen of the bases, in the case of the dog rendered immune to ricin, is a trifle lower; the difference is so slight, however, and variation among normal organs is seen to be so great, that it is doubtful if the relation between that part of the protein yielding the bases, and the protein as a whole was disturbed by the ricin treatment. It is recalled, in the case of the immune horse, that the nitrogen of the bases was slightly higher than the normal values (Table II).

The arginin and lysin from the liver of the phlorhizin dog show a remarkably wide variation. The nitrogen of the arginin is low, being 4.17 per cent of the total nitrogen; on the other hand, the nitrogen of lysin is high, being 7.42 per cent of the total nitrogen. These results suggest, at least, some connection in their variation, since the sum of these values, 11.59, stands to the nitrogen of the three bases, 13.39, in a ratio quite closely agreeing with normal values. The nitrogen of the three bases is seen to be about 2 per cent lower than the value established for the normal.

The alterations in the composition of the protein substances after phlorhizin poisoning appear to be less in extent than after phosphorus poisoning. The average results from the livers of the phosphorized dogs indicate a considerably greater breaking down of those groups yielding the bases, the nitrogen of the bases being 9.31 per cent of the total nitrogen. (See footnote 1, p. 120).

In the case of the dog which fasted, whose liver appeared normal by microscopical examination, the nitrogen of the three bases is about 2.5 per cent lower than the normal; the nitrogen of the arginin is also somewhat lower than that of the lysin. The relation between the sum of these values, 11.07, to the nitrogen of the three bases, 12.86, is seen to be the same as in the case of the phlorhizin dog and the ricin dog, and quite similar to the normal values, suggesting again a mutual relation between the arginin and lysin values.

The percentage of dry substance from the liver of the dog which fasted, is not unlike the normal, but the nitrogen content is about 1 per cent higher. The high solids and low nitrogen in the case of the phlorhizin dog is, of course, attributable to the excess of fat.

The results from the pathological livers presented in the table suggest at least a slight molecular rearrangement, resulting in a slight diminution of those atomic groups of the molecule from which the bases are derived, since the average nitrogen of bases from the livers of the pathological animals is some 2 per cent lower than the normal value; the difference, however, is so slight, that more than a suggestion of a change of this nature is unwarrantable. No evidence in any of the cases is at hand showing an absolute loss of nitrogen.¹

¹ Through the kindness of Dr. Levene, I received a liver from a dog whose pancreas had been removed three weeks previous to death. Masses of the liver had been thrown into alcohol, and it was some time afterward that the liver reached the laboratory. Of course conditions existed favorable for autolysis within these liver masses beyond the point of penetration of the alcohol.

The alcohol was decanted off and evaporated at a gentle temperature and the residue intimately mixed with the rest of the finely divided tissue, and homogeneous portions taken for drying and for the estimation of total nitrogen in the dry substance; other portions were taken for the estimation of the bases.

During the analysis, the fraction containing the arginin and histidin as carbonates in neutral solution was inadvertently allowed to stand without acidifying. When the work was resumed, several days later, a considerable growth of mould was found to have formed.

Inasmuch as this liver was of such an unusual character I report the results with considerable hesitation, without attempting to interpret them.

NITROGEN OF DRY SUBSTANCE = 4.66 PER CENT.

Total nitrogen	=	100.	Nitrogen of the three bases	=	100.
Nitrogen of arginin	=	2.62	" " arginin	=	41.8
" " lysin	=	3.33	" " lysin	=	53.0
" " histidin	=	.32	" " histidin	=	5.2
		<hr/> 6.27			<hr/> 100.0

Comparing with the results in Table III, the nitrogen of dry substance is seen to be some 60 per cent lower than the average normal value.

The nitrogen of the bases is seen to have fallen off over half, the nitrogen of each of the individual bases being lower, but that of histidin especially so. It is a question whether it is safe to draw the inference from these results that the changes in the liver due to the diabetic process are as profound as they would seem to indicate. They are probably modified to some extent by autolytic changes after the liver was taken from the dog, and by contamination with the mould. Nevertheless it seems improbable that these disturbing factors can more than partially account for the extreme results recorded here.

V. KIDNEY OF THE DOG.

In presenting the results from normal livers of different individuals of the same animal species, allusion has been made to the fact that the occurrence of the bases is subject to considerable variation. To eliminate the factor due to individual differences, experiments were planned to obtain from the same animal a normal and a pathological specimen for examination. This is readily possible in the case of the kidney and muscle. In the case of the kidney it was intended to determine the influence, on the composition of this organ of the degenerative changes in the renal epithelium which follow a temporary deprivation of the blood supply. An error was made, however, in making the period of the experiment so short as to hardly afford time for the development of well-defined nutritional changes in the protoplasm of the kidney.

In order to get sufficient material for the analysis, four normal dogs were taken. One kidney from each was removed, and at the same time the blood supply was shut off from the other kidney. With the exception of Dog III (Table IV) which lived only three hours, the dogs were killed at the end of eight or nine hours by bleeding. After removing the blood as much as possible without washing through the blood vessels, the eight kidneys were individually examined for total solids and nitrogen. The results are recorded in Table IV, Part 1.

As seen by the table each of the injured kidneys weighed heavier than the corresponding normals; the percentage of dry substance was less in the injured kidneys than in the normals, with the exception of the animal which died at the end of three hours. The nitrogen of the dry substance is seen to be a trifle less in the injured kidneys with one exception than in the corresponding normal kidneys.

The results upon the combined kidneys, normal and injured, are found in Table IV, Part 2.¹

In examining the results, those from the injured kidneys are seen to vary so little from the normal that no significance can be

¹ The results for total solids and total nitrogen are calculated from the results given in Table IV, Part 1, based on the individual amounts of organ taken.

TABLE IV. PART I.

NATURE OF EXPERIMENT.		WEIGHT OF EACH KIDNEY. GMS.		AMOUNTS TAKEN FOR ESTIMATION OF BASES. GMS.		DRY SUBSTANCE IN 100 PARTS OF MOIST TISSUE.		DRY SUBSTANCE = 100	
KIDNEY.								TOTAL NITROGEN.	
Dog.		Normal.	Injured.	Normal.	Injured.	Normal.	Injured.	Normal.	Injured.
I	18.	22.	15.7	20.	20.94	19.14	12.47	12.33
II	15.5	16.5	13.2	14.4	22.32	21.49	11.60	12.19
III	36.	45.	34.4	42.	19.86	23.41	12.46	11.70
IV	24.	28.	22.	24.3	23.59	20.75	12.00	11.47
Total	93.5	111.5						

TABLE IV, PART 2.

NATURE OF EXPERIMENT.		Dog.	Case.	Dry substance in 100 parts of moist tissue.	DRY SUBSTANCE \approx 100.				TOTAL NITROGEN OF DRY SUBSTANCE \approx 100.				TOTAL NITROGEN OF BASES \approx 100.				
KIDNEY.	Total nitrogen.				NITROGEN OF BASES.			NITROGEN OF BASES.			NITROGEN OF BASES.			NITROGEN OF BASES.			
					Arginin.	Lysin.	Histidin.	The three bases.	Arginin.	Lysin.	Histidin.	Arginin and Lysin.	The three bases.	Arginin.	Lysin.	Histidin.	
I-IV	Normal.			21.43	12.17	0.511	0.737	0.306	1.554	19.6	0.52	5.1	10.24	12.75	32.94	7.51	9.6
I-IV	Injured.			21.65	11.84	0.530	0.641	0.241	1.414	18.5	4.2	2.04	9.90	11.94	37.54	5.41	7.1

attached to them. The shutting off of the blood supply was not of sufficiently long duration to bring about any alteration. The results must be interpreted therefore as negative.

The most striking feature among the results, as previously intimated is the relatively low value for arginin. This is true for the normal as well as for the injured organs, and in a trifle greater degree. The lysin values agree fairly well with values obtained from other tissues (Table I), but the histidin values are relatively higher.¹

The total nitrogen of the three bases is in fair agreement, but slightly lower than the corresponding value from other organs.

VI. MUSCLE OF THE DOG.

It seemed worth while to plan experiments to determine whether the atrophic changes induced in muscles by section of their motor nerves are of a sufficiently profound nature to induce definite alterations in the relationships of the bases derived from the tissue proteins. Such changes even if relatively slight when compared with the values from the corresponding normal muscles of the same dog would have the greater significance for the factor of individuality would be eliminated, as already stated.

The sciatic and anterior crural nerves on the same leg of a normal dog weighing 11.5 kilos were resected. The wounds healed completely. After thirty-one days, the dog was killed by bleeding and the corresponding gastrocnemius and soleus muscles carefully dissected from each leg. The normal gastrocnemius muscle weighed 45 grams, the corresponding degenerated muscle weighed 34 grams. The normal soleus muscle weighed 18 grams, and the corresponding degenerated muscle weighed 10.7 grams. The normal muscles together therefore weighed 63 grams, while the degenerated muscles weighed 44.7 grams, a shrinkage of 29 per cent.

The two sets of muscles presented some difference of appearance to the naked eye, but the microscopical examination showed nothing in any way abnormal in either. Equal weights of each set were taken for analysis, and the two were carried through side by side. The results are reported in Table V.

¹ See footnote, p. 130.

TABLE V.

NATURE OF EXPERIMENT.		Dry substance in 100 parts of moist tissue.		DRY SUBSTANCE = 100.				TOTAL NITROGEN OF DRY SUBSTANCE = 100.				TOTAL NITROGEN OF BASES = 100			
MUSCLE.				NITROGEN OF BASES.				NITROGEN OF BASES				NITROGEN OF BASES.			
Dog.	Case.			Total nitrogen.	Arginin.	Lysin.	Histidin.	The three bases.			Arginin.	Lysin.	Histidin.		
I II {	Normal.....	26.47	12.68	1.308	0.935	0.402	2.65	10.32	7.37	3.17	17.69	20.86	49.5	35.3	15.2
	Normal.....	30.09	12.32	1.085	1.171	0.334	2.59	8.61	9.51	2.71	18.12	20.83	41.3	45.7	13.0
	Degenerated.....	27.86	12.02	0.995	1.058	0.309	2.36	8.28	8.81	2.57	17.09	19.66	42.1	44.8	13.1

In the degenerated muscle there is noticed a diminution of 2.2 per cent in solids and a slight diminution in the nitrogen of solids.¹ The nitrogen of the three bases in the normal muscle is 20.83 per cent of the total nitrogen; in the degenerated muscle it is 19.66 per cent, a drop of 1.17 per cent. Each individual base obtained from the degenerated muscle is a trifle lower than the corresponding base from the normal muscle, but the relation of the bases to one another is very similar as seen in the last three columns of the table. The relation of arginin plus lysin to histidin is identical in the two cases. What variation there is exists between the arginin and the lysin, but this is probably within the limits of experimental error.

A determination of the bases was also made on the muscle of another dog. He was a normal, well nourished animal, weighing 8 kilos. He was killed by bleeding, and the thigh muscles from both legs were taken for analysis. The results are recorded in the first series in Table V. These results, taken in connection with those from the normal muscle of the other dog, afford a good illustration of the degree of variation among the bases which one may expect to find from the examination of the same organ from different animals of the same species. While the variation among the bases is greater in the case of the normal muscles from the two animals, than in the case of the normal and degenerated muscles of the same animal, still the latter series of results are the more significant for the reason that in this case all factors relating to differences of individuals are eliminated. It is probably true that a greater insight into the changes affecting the protein molecule under varying conditions is to be hoped for when the study, if applied to animal proteins, is so planned as to eliminate those differences which normally occur in the difference due to the individual.

In comparing the results from the normal muscles of the two dogs, a close agreement is seen to exist between the nitrogen of

¹ An increase of glycogen in muscle after section of its motor nerves was observed by Chandelon (*Arch. f. d. ges. Physiol.*, xiii, p. 626). The slightly lower nitrogen of dry substance in the case of the degenerated muscle might indicate some change of this nature, though the percentage of dry substance is less in the moist tissue of the degenerated muscle than in the normal.

the three bases; in one case it is 20.86 per cent of the total nitrogen, in the other, 20.83 per cent. The percentages of nitrogen of arginin plus lysin in the two cases is 17.69 and 18.12. While the relation of the three bases to one another is fairly constant in the two series, the greatest variation exists between the arginin and lysin.

It is a matter of interest that the protein of muscle when compared with the protein of liver or kidney, as already indicated in Table I, is relatively richer in those atomic groups which separate as bases on hydrolysis.

VII. LIVER OF MAN.

In connection with the special study on the normal as well as the experimentally induced pathological organs of the dog and horse, an extension of the study to include pathological, and if possible, normal specimens of tissue from man seemed desirable. The opportunity was presented from time to time of obtaining pathological samples of liver tissue from patients dying from various diseases in which the liver changes were prominent, and two specimens were obtained from healthy individuals dying from accident.

In Table VI, the observations on normal and pathological human livers are recorded.

In the examination of the results from human organs, the influence of outside factors which are under only partial control must be taken into account, chief among which are the possible changes the organ may undergo before it is subjected to chemical analysis. Of course this is a variable quantity but an endeavor was made to minimize this factor as much as possible. The bodies as a rule are kept on ice before the autopsy and the delay between the autopsy and the time the organ was brought into contact with strong sulphuric acid was usually short. The changes due to autolysis, or bacterial activity, were probably slight—nevertheless some changes of this kind must be considered as possible.

In the normal livers (I and II) the agreement between solids and total nitrogen is seen to be very close. Allusion has already been made to the widely separated values for the three bases,

the nitrogen of the one being 12.91 per cent of the total nitrogen, and of the other, 18.08 per cent. The mutual relation between the three bases is, however, very similar, as seen in the last three columns of the table. The difference between the bases, amounting to over 5 per cent in livers apparently so comparable, is interesting as suggesting how variable may be the different groups which make up the cell proteins, with a tendency, however, toward the maintenance of a constant relation among certain of the groups.

The cirrhotic liver (III) was most altered structurally. The nitrogen content of the solids is seen to be about 1 per cent below the average. Of course in any consideration of chemical alterations in livers which are the seat of cirrhosis, it must be remembered that the connective tissue elements being unduly increased in proportion to the parenchymatous elements, there may be a fall in total nitrogen of the dry substance which is dependent on this admixture of connective tissue cells with specific liver cells. We have no means of knowing in such cases to what extent the low nitrogen is due to a fall in the nitrogen of the parenchymatous cells themselves. The nitrogen of the bases is also low, being 11.17 percent of the total nitrogen. This drop in the nitrogen of bases, being some 4.3 per cent below the average normal value is attributable both to the arginin and lysin, the histidin being nearly equal to the average normal value. If we compare the values from the cirrhotic liver with the lower series of values from the normal livers (I), the values of the former are still seen to be appreciably lower. Thus the percentage of nitrogen of the three bases of the normal is 12.91; of the cirrhotic, it is 11.17, a drop of 1.74. Comparing the corresponding values in the case of arginin plus lysin, the drop is seen to be 1.92, that is, the loss is due to the arginin and lysin. The relatively low arginin and lysin compared to histidin is brought out in the last columns of the table.

It would appear from the above results that in the condition of cirrhosis, processes are at work in the liver protein of such a nature as to cause some disturbance of the normal nitrogenous equilibrium, slight in amount, whereby those groups in the molecule yielding arginin and lysin become diminished. Whether the nitrogen of those groups is lost from the molecule, or remains in the molecule in some other form it is difficult to say, since the

TABLE VI.

NATURE OF EXPERIMENT.		Dry substance in 100 parts of moist tissue.	DRY SUBSTANCE = 100.		TOTAL NITROGEN OF DRY SUBSTANCE = 100.		TOTAL NITROGEN OF BASES = 100.								
Human.	LIVER.		NITROGEN OF BASES.		NITROGEN OF BASES.		NITROGEN OF BASES.								
			Total nitrogen.	Arginin.	Lysin.	Histidin.	The three bases.	Arginin.	Lysin.	Histidin.					
I	Normal.....	25.29	11.79	0.661	0.653	0.207	1.52	5.61	5.54	1.76	11.15	12.91	43.6	42.9	13.5
II	Normal.....	24.86	11.98	0.977	0.917	0.271	2.168	16.7	6.62	2.26	15.82	18.08	45.1	42.4	12.5
Av.	I, II.....	25.08	11.89	0.819	0.785	0.239	1.846	8.86	6.60	2.01	13.48	15.49	44.4	42.6	13.0
III	Cirrhosis.....	26.23	10.81	0.524	0.474	0.210	1.214	8.54	3.81	1.94	9.23	11.17	43.4	39.2	17.4
IV	Acute yellow atrophy.....	20.69	13.60	0.850	0.891	0.320	2.066	25.6	2.55	2.35	12.80	15.15	41.3	43.2	15.5
V	Acute streptococcus septicæmia.....	22.42	12.72	0.654	0.625	0.245	1.525	15.4	9.21	1.93	10.07	12.00	42.9	41.0	16.1
VI	Puerperal eclampsia	25.97	12.36	0.793	0.662	0.210	1.666	6.42	5.36	1.70	11.78	13.48	47.6	39.8	12.6
VII	Yellow fever							5.74	6.37	2.07	12.11	14.18	40.5	44.9	14.6
VIII	Yellow fever							7.36	7.85	2.94	15.21	18.15	40.5	43.3	16.2
IX	Miliary tuberculosis of the lungs.....	20.86	12.94	0.949	1.084	0.489	2.527	33.8	38.3	78.15	71.19	49.37	6.43	0.19	4
X	Infant.....	20.33	12.54	0.308	0.665	0.233	1.212	45.5	5.30	1.86	7.75	9.61	25.5	55.2	19.3

- I. Normal man. Day laborer, *act.* about 25 years; short, stocky, well nourished, weighing about 140 pounds Killed by accident. Autopsy three hours after, and three and three-quarters hours later the finely divided liver tissue was brought in contact with strong sulphuric acid. Histological examination of tissue revealed nothing abnormal.
- II. Normal boy. *Act.* about 14 years; only moderately well nourished, weighing about 90 pounds. Killed by accident. Autopsy nine hours after death and three hours later the liver tissue was started for analysis. The liver was anemic but nothing abnormal was shown by the microscopical examination. The liver tissue was in an excellent state of preservation.
- III. Man, *act.* 26; chronic hypertrophic cirrhosis of the liver, with jaundice. Liver much enlarged, with finely granular surface. On section many lobules present yellow appearance, the distribution suggesting diffuse fine cirrhosis. Microscopic examination showed cirrhosis to be of high grade and of general distribution. Connective tissue was throughout the lobules. Fatty degeneration of the cells was very marked. Relation of time of autopsy to time of death was not given.
- IV. Boy, *act.* 14; well nourished. Acute yellow atrophy of the liver. Autopsy seventeen hours after death. Liver 27.5 ounces, flabby, friable and soft, considerably wrinkled. Section shows coloring and mottling as on surface, as if the liver substance had shrunk beneath the peritoneal covering, forming linear furrows, especially on the left side. Surface color was dark red with irregular mottling of a dark yellowish brown. Histological examinations showed areas of moderate fatty degeneration scattered throughout the section.
- V. Woman, *act.* 29. Acute streptococcus septicemia. Died from septic peritonitis two weeks after childbirth. Temperature remained about 99° till death. Autopsy twenty-four hours after death. The liver showed subcapsular congestion and marked cloudy swelling. Histological examination showed scattered areas at the periphery of the lobules with some fatty degeneration.
- VI. Woman, *act.* 23. Puerperal eclampsia with acute parenchymatous nephritis. Autopsy two hours after death. Liver showed marked cloudy swelling. Histological examination revealed nothing abnormal.
- VII and VIII. Cases of typical yellow fever. From a communication from New Orleans it was learned that the notes on the cases had been mislaid.
- IX. Girl, *act.* 19. Miliary tuberculosis of lungs. Perforation of pleura. Tuberculous enteritis with ulcers. Autopsy thirty-six hours after death. Liver firm, surface smooth. Section congested lobules, faintly marked. Histological examination showed liver to be moderately fatty and congested.
- X. Normal infant, one hour. Cerebral hemorrhage. Autopsy twenty-four hours after death. Liver anemic. Histological examination shows congestion. Fine connective tissue among the liver cells is abundant. An occasional nucleus among the liver cells does not stain.

value for nitrogen of solids is influenced by a somewhat disturbing factor—the connective tissue—as already mentioned.

The fact that the histidin apparently remains unchanged is interesting. This certainly suggests a greater stability or resistance to breaking down influences of that portion of the molecule from which histidin springs than is true of the other groups under consideration. It has been suggested that this apparent stability of the histidin complex is associated with its cyclic structure.

In acute yellow atrophy it is well known that amino acids, such as leucin and tyrosin find their way into the urine, and there is no reasonable doubt that these bodies are derived under these conditions from the cells of the liver. One would expect to find under these conditions a low nitrogen content of solids, unless the products from the breaking down of the protein were suffered to accumulate in the liver. But if there were an accumulation of this nature, one would expect the nitrogen of the bases to be relatively low. By inspection of the results it is noticed that the nitrogen of solids is higher than the normal, suggesting an accumulation of nitrogenous products. At the same time the nitrogen of the bases is seen to be well within range of the normal values, showing at least no apparent diminution compared to the total nitrogen.

Of course the high nitrogen of solids could be due to a breaking down of the liver elements with an accumulation or retention of some of the nitrogenous products, or a breaking down of the nitrogenous elements without necessarily a retention of the products, but accompanied by a more rapid breakdown of the non-nitrogenous elements. The lack of low values found for the bases would harmonize with this latter view. This example illustrates how profound may be the histological changes in an organ, while the protein substances as far as may be shown by the analysis, maintain their approximate composition.

The case of acute streptococcus septicæmia (V) shows greater variation from the normal than is true in the case of acute yellow atrophy. The total solids are somewhat lower than the normal. The nitrogen of bases, being 12.0 per cent of total nitrogen, is some 3.5 per cent below the average normal. The histidin nitrogen agrees closely with the normal value; the decrease of nitrogen of the bases is seen to be due to the lower values for arginin and lysin.

If compared with the lower normal values of Series I, instead of the average normal values, the percentage of nitrogen of the three bases, in the case of the acute septicæmia, is seen to be 0.91 per cent lower; the value for the nitrogen of arginin and lysin is 1.08 lower.

This example is seen to resemble the case of cirrhosis in regard to the variation of the bases compared with the normal values, except that it is not so marked, illustrating again the apparent resistance of that atomic group which yields the histidin to those influences which seem to cause slight breaking down of the other groups from which the bases are derived. There does not appear to be any actual loss of nitrogen from the molecule; the lower arginin and lysin values suggest rather a different atomic arrangement. But in comparison with the lower normal values from Series I the differences are so slight that their interpretation is only suggestive.

In the case of puerperal eclampsia (VI) the nitrogen of the bases is seen to be 13.48 per cent of the total nitrogen, being 2 per cent lower than the corresponding average normal value, the difference being somewhat evenly distributed among the bases. If, however, the results are compared with those of the lower of the normal values (I), they are found to be such as afford no evidence that the liver protein in the condition of puerperal eclampsia is altered.

Through the kindness of Dr. Flexner, two livers were obtained from patients who had died of yellow fever (VII, VIII). The livers were sent from New Orleans preserved in 5 per cent sulphuric acid; the cases were said to be typical. I am unable to report any microscopical examination of the tissues. A determination of total solids of the moist tissue was unfortunately impossible, hence the information from the analyses is relatively limited.

In one case (VII) there is some diminution of nitrogen of bases compared with the average normal values; in the other, however, there is an increase, the average of the two being 16.16, agreeing closely with the normal value, 15.49, indicating that the relation of the groups yielding bases to the total nitrogen of the protein is maintained in the condition peculiar to yellow fever. The mutual relation of the bases also resembles the normal, the greater variation being with the arginin and histidin.

It is unfortunate that we are unable to state whether the nitrogen of dry substance of the liver was reduced in these cases, and whether the dry substance of the liver bears the normal relation to the moist tissue. The opportunity was welcome to make analyses on the liver tissue from yellow fever and it was thought that the most profound changes of which the liver is susceptible during life might be expected to be present here owing to what is known of the gross and microscopical appearance in such cases. All that can be said in regard to our cases is that the normal individual relation between the bases has been maintained.

In an effort to get specimens in which little or no connection was thought probable to exist between the cause of death and the nature of the liver, in the effort that is, to get normal livers, one from a case of miliary tuberculosis of the lungs was obtained (IX). Histological examination showed it to be somewhat abnormal, and unfortunately considerable time elapsed before the autopsy was made. It seemed worth while, however, to carry through the analysis.

The content of solids is seen to be low, and the nitrogen of solids is somewhat higher than the normal. The most striking result of the analysis is the high nitrogen of bases, being 19.49 per cent of the total nitrogen. It may be that this is within the limit of normal variation. The highest result found from examination of the normal tissue was 18.08. The relation of the arginin to histidin varies considerably from the normal as seen in the last columns of the table. This may be due in part to experimental error.¹

The peculiar results obtained from the liver protein of an infant (X) I am unable to interpret. The autopsy was made after twenty-four hours, and the liver did not reach the laboratory until the following day. In the meantime, however, it was on ice. The percentage of dry substance is seen to be somewhat low and the percentage of the nitrogen of dry substance is possibly a little high. The most striking feature the analysis brought out was the markedly low arginin when compared with the results from the adult human liver. The nitrogen of the bases was

¹ See note, p. 130.

found to be 9.61 per cent of the total nitrogen, the lowest of the adult normal livers being 12.91 per cent. This difference is largely due to the low nitrogen of arginin, the lysin and histidin nitrogen content being not unlike the values found in the adult normal livers. The nitrogen of arginin is seen to be only 2.45 per cent of the total nitrogen.

I hope ultimately to have opportunity to determine whether a relatively lower arginin moiety is actually a feature of infantile as compared with adult tissue.

VIII. SUMMARY.

Attention has been drawn to the presence of certain modifying factors which must be taken into account in interpreting our results as indicating a definite relation between certain atomic groups to one another and to the molecule of which they form a part—factors such as the presence of an appreciable amount of non-protein nitrogen in the tissue, nature of the method, etc.—and cognizance is taken of these factors in summarizing the results reported.

Considerable variation in regard to the proportion of those groups taken as a whole, which give rise to arginin, lysin and histidin, contained in the tissue proteins was shown to exist among organs of the same kind from different individuals of the same species. The mutual relation of the three groups was seen to vary between narrower limits.

In the light of the apparently normal variations above alluded to, the relation of these groups to the entire molecule was seen to be quite constant in the protein obtained from the same organ of the various animal species—man, dog, horse and fish; the widest departure being observed in the case of the fish.

A less constant relation between those groups yielding the bases and the whole molecule was found when protein from different normal organs from the same animal species was examined, as the muscle liver and kidney of the dog. The number of groups giving rise to the bases was relatively large in the proteins of the muscle and small in the proteins of the kidney.

In regard to the results obtained from the study of the possible alterations in the protein substances for various pathological

organs from different animal species as revealed by the difference of relation compared to the normal, it was found that the response of the protein molecule to influences causing in some instances most profound histological changes was as a rule comparatively slight. It was found, for instance, that the composition of the protein from liver tissue showing such gross and histological changes as accompany acute yellow atrophy was not essentially changed as far as the bases were concerned.

In those cases where the changes in the protein composition were somewhat noticeable, with apparently one exception, they involved a diminution of that portion of the protein molecule from which the bases originate. This diminution for the most part appeared to be the result of a rearrangement without material loss of nitrogen. The results obtained from the various conditions studied seem, upon the whole, to indicate a tendency toward a diminution of those groups yielding the bases, rather than an increase.

In regard to the relation of the individual groups to the entire molecule in those cases where there had apparently been some change in the molecular structure of the protein, resulting in a diminished number of the groups from which the bases spring, it was found more often than otherwise that the diminution came from those groups yielding arginin or lysin or both, with perhaps evidence of a less resistance in the case of the group yielding arginin. Of the three groups, there seems to be some evidence that the group yielding histidin is the least easily influenced by the degenerative changes.

Among the twenty-one estimations reported in the present paper, seven were made upon tissues accepted as normal, the remainder upon tissues in supposedly various degrees of alteration. Of these, some showed very marked histological changes, others none, or very slight. In regard to the changes of protein composition from the interpretation of the results of the analyses of these thirteen supposedly altered tissues the results from five analyses were unmistakably within the limits of normal variation, and the protein structure pronounced unaffected. Two indicated no change as far as the results obtained could show; one showed a slight tendency of alteration toward an increase of those groups yielding the bases, while the remaining five showed

a tendency toward a diminution of those groups. The slight diminution of the groups yielding bases was interpreted as indicating a rearrangement of nitrogen in the protein molecule rather than a loss of nitrogen from it.

The interpretation of the results throughout the paper has been made with some hesitation, being little more than suggestive owing to the differences being, as a rule, so slight, and the differences of normal variation by no means inconsiderable.

FURTHER OBSERVATIONS ON THE INHIBITING EFFECT
OF FLUORIDES ON THE ACTION OF LIPASE,
TOGETHER WITH A METHOD FOR THE
DETECTION OF FLUORIDES IN
FOOD PRODUCTS.

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Loevenhart and Peirce¹ have recently studied the inhibiting effect of sodium fluoride on the hydrolysis of various esters by extracts of different organs. They found that extremely small amounts of the fluoride inhibit the action of clear liver extract on ethyl butyrate and other esters to a remarkable extent. Ammonium fluoride and hydrofluoric acid act quite similarly to sodium fluoride. The idea suggested itself to us that a method for the detection of small quantities of fluorides might be based on these observations. We have taken occasion also to extend the previous observations in regard to the mechanism of the inhibition and this part of our work will be presented first.

PART I.

FURTHER OBSERVATIONS BEARING ON THE FLUORIDE INHIBITION.

Loevenhart and Peirce found that the extent of the fluoride inhibition is determined principally by the acid moiety of the ester and is largely independent of the alcohol from which the ester is derived. They found that with the esters of the fatty acids the fluoride inhibition decreases with increasing molecular weight of the acid from which the ester is derived. Thus esters of acetic acid show a greater inhibition than corresponding esters of butyric acid and with the higher fats the inhibition is far less than with butyric esters. In order to see whether there is a regular decrease in the fluoride inhibition as we ascend the fatty

¹ This *Journal*, ii, p. 397, 1907.

acid series we have extended these observations to the following esters; the ethyl esters of acetic, propionic, butyric and valeric acids, the methyl esters of caprylic and lauric acids. We have limited ourselves in this work to hepatic lipase of the pig. The quantities of these esters employed in each experiment were as follows: Ethyl acetate, 0.1747 gram; ethyl propionate, 0.2082 gram; ethyl butyrate, 0.23 gram; ethyl valerate, 0.261 gram; methyl caprylate, 0.316 gram; methyl laurate, 0.425 gram.¹ In our technique we have not deviated from that used in the previous work. Toluene was used in all experiments as the antiseptic.

SERIES I. Duration of experiments, 20 hours. Temperature, 37°. One cc. of a 10 per cent clear liver ext act and 4 cc. of water, or 3 cc. of water and 1 cc. of sodium fluoride solution were used in each experiment. In the fluoride experiments this salt was present in a concentration of 1 : 100,000.

No. of experiment.	Ester.	cc. of $\frac{N}{20}$ NaOH required.		Inhibition, per cent.
		Water.	NaF.	
1	Ethyl acetate	7.15	0.58	91.9
2	Ethyl propionate	10.88	1.2	89.0
3	Ethyl butyrate	11.57	2.4	79.3
4	Ethyl valerate	7.92	2.3	71.0

SERIES II. Duration of experiments, 17 hours. Temperature, 38.5°. Conditions otherwise identical with Series I.

No. of experiment.	Ester.	cc. of $\frac{N}{20}$ NaOH required.		Inhibition, per cent.
		Water.	NaF.	
1	Ethyl butyrate	10.4	3.3	68.6
2	Methyl caprylate	7.1	2.9	59.2
3	Methyl laurate	0.8	0.37	53.7

SERIES III. One cc. of turbid 10 per cent liver extract was used in each experiment instead of the clear extract. Otherwise the conditions were identical with those of Series II. At the end of the experiments

¹ The reason for using the esters in these quantities has been given. See this *Journal*, ii, pp. 398 to 399, 1907. The technique which we have employed is also there described.

alcohol and phenolphthalein were added and the titrations were made with alcoholic potassium hydroxide.

No. of experiment.	Ester.	cc. of $\frac{N}{20}$ NaOH required.		Inhibition, per cent.
		Water.	NaF.	
1	Ethyl butyrate	12.75	3.62	71.6
2	Methyl caprylate	17.75	7.85	55.8
3	Methyl laurate	1.75	1.02	41.7

These experiments show that there is quite a regular decrease in the fluoride inhibition with increasing molecular weight of the acid from which the ester is derived.

Loevenhart and Peirce found that the greater the amount of enzyme acting the less was the percentage inhibition noted in the fluoride experiments. It occurred to us that some admixture present in the extract entirely distinct from the enzyme might protect the latter against the fluoride. In order to throw some light on this point experiments were performed in which a quantity of boiled extract was added to some of the same extract which had not been boiled.

SERIES IV. Duration of experiments, 16 hours. Temperature, 38°. One cc. of 10 per cent clear liver extract and 0.26 cc. of ethyl butyrate were used in each experiment. The total volume in each experiment was 5 cc. In experiments 1 and 3, 4 cc. of water or boiled extract were employed, while in experiments 2 and 4, 3 cc. of water or boiled extract were used together with 1 cc. of sodium fluoride solution, the latter acting in a concentration of 1 : 50,000.

No of experiment.		cc. of $\frac{N}{20}$ NaOH required.	Inhibition, per cent.
1	Extract, water	8.0	
2	Extract, water, fluoride	1.05	88.3
3	Extract, boiled extract.	9.4	
4	Extract, boiled extract, fluoride	1.85	80.3

These experiments show that while the boiled extract protects to a slight extent against the fluorides, this protective action is insufficient to explain the results of Loevenhart and Peirce and their contention that there exists a quantitative relationship

between the amount of enzyme acting and the fluoride inhibition is valid as far as our experiments indicate.

The protective action of the sodium salts of the fatty acids against the inhibiting action of the fluorides. It was frequently noted that acid develops with surprising rapidity after titrating the tubes containing the fluoride. This led Loevenhart and Peirce to study the effect of keeping the tubes as nearly neutral as possible by adding the alkali drop by drop as the acid developed. Under these conditions it was found that the inhibiting action of the fluoride is considerably diminished. In order to explain this fact it was assumed that as the tubes become more acid hydrofluoric acid is developed and that this substance probably inhibits the enzymic hydrolysis to a greater extent than sodium fluoride. Several facts led us to believe that this is not the correct explanation and it occurred to us to study the effect of the sodium salts of the fatty acids on the fluoride inhibition. This work soon proved that these salts have a marked protecting action against the fluoride as is brought out in the following tables:

SERIES V. Duration of experiments, 21 hours. Temperature, 37°. One cc. of 10 per cent clear liver extract and 0.26 cc. of ethyl butyrate were used in each experiment. The total volume in each experiment was 5 cc. and the concentration of the sodium salts in the total volume is given in the table.

Concentration of sodium butyrate.	cc. of $\frac{N}{20}$ NaOH required.		
	Water.	NaF	
		1 : 50,000.	1 : 500,000.
0.0	9.2*	0.9	3.17
$\frac{N}{10}$	10.6	5.25	9.35
$\frac{N}{25}$	10.38	3.93	8.33
$\frac{N}{50}$	10.25	2.93	6.85

The percentage acceleration or inhibition calculated in reference to the tube which contained neither sodium butyrate nor fluoride (viz: the one marked * in the above table) is shown in the following table. The sign, +, indicates acceleration, while — indicates inhibition.

Concentration of sodium butyrate.	Per cent acceleration or inhibition.		
	Water.	NaF	
		1:50,000	1:500,000
0.0		-90.2	-65.5
$\frac{N}{10}$	+15.2	-42.9	+ 1.6
$\frac{N}{25}$	+12.8	-57.3	- 9.5
$\frac{N}{50}$	+11.4	-68.2	-25.5

This table shows that sodium butyrate accelerates the action of the enzyme in the absence of the fluoride. This accelerating action does not increase proportionately with increasing concentration of the butyrate. The butyrate when acting in $\frac{N}{10}$ solution decreases the inhibition by the fluoride, 1:50,000, to less than one half and completely neutralizes the effect of the fluoride acting in a concentration of 1:500,000. A solution of sodium fluoride 1:500,000 expressed in terms of normal is $\frac{N}{210000}$. Hence in order to completely neutralize the fluoride inhibition the sodium butyrate must be present in a molecular concentration 2100 times as great as that of the fluoride. In a concentration of $\frac{N}{25}$, sodium butyrate materially decreased the fluoride inhibition. It is also to be noted in this table that the accelerating action of the different strengths of sodium butyrate in the absence of fluoride does not run parallel to the protective action against the fluoride. Thus when acting in $\frac{N}{25}$ solution the accelerating effect is very little greater than in $\frac{N}{50}$ and yet the former exercises a much more powerful protecting effect against the fluoride. This fact opposes the view that in this interesting antagonism we have merely the summation effect of two factors, one of which tends to accelerate the reaction and the other to retard it. It should be mentioned that when the fluoride acts in a concentration of 1:10,000 sodium butyrate in the concentrations here used has very little neutralizing action.

In regard to the mechanism of the antagonism between the fluoride and butyrate it occurred to us that since butyric acid greatly inhibits the hydrolysis of ethyl butyrate, sodium butyrate might accelerate the reaction by driving back ionization of the butyric acid, produced in the course of the hydrolysis, in consequence of the common butyric ion. Again, there might be

found a certain correspondence between the ester and the sodium salt of the same fatty acid from which the ester is derived, or, in other words, it might be found that sodium butyrate would accelerate the hydrolysis of ethyl butyrate and in the presence of fluoride, protect, better than sodium acetate or propionate. We have, therefore, made a study of the effects of the sodium salts of acetic, propionic, butyric and isovaleric¹ acids upon the hydrolysis of the esters of the same acids both in the presence and absence of sodium fluoride. The results are given in the following tables:

SERIES VI. *The effect of the sodium salts of the fatty acids on the hydrolysis of the esters in the absence of sodium fluoride.* Duration of experiments, 19 hours. Temperature, 37°. One cc. of 10 per cent clear liver extract was used in each experiment. The total volume in each case was 5 cc. The salts all acted in a concentration of $\frac{N}{10}$. In the table the column marked NaOH indicates the number of cc. $\frac{N}{50}$ NaOH required to neutralize the acid produced. In the corresponding column marked per cent is given the per cent acceleration (marked +) or the per cent inhibition (marked -).

	Ethyl acetate.		Ethyl propionate.		Ethyl butyrate.		Ethyl valerate.	
	NaOH	Per cent	NaOH	Per cent	NaOH	Per cent	NaOH	Per cent
Water.....	7.15		10.88		11.57		7.92	
Sodium acetate....	11.38	+ 59.5	15.78	+ 45.0	15.1	+ 30.5	9.32	+ 17.7
Sodium propionate	5.98	- 16.4	11.5	+ 5.7	13.45	+ 16.2	8.17	+ 3.2
Sodium butyrate..	5.55	- 22.4	10.53	- 3.2	12.62	+ 9.0	7.95	+ 0.4
Sodium isovalerate	5.4	- 24.5	10.05	- 7.6	12.4	+ 7.2	7.9	- 0.3

This series of experiments shows (1) that the sodium salt of the fatty acid from which the ester is derived has no special accelerating action and hence the view that the accelerating action depends on the presence of a common ion in the salt and the acid resulting from the hydrolysis is untenable. (2) That for a given ester the accelerating action of the sodium salt decreases as we ascend in the fatty acid series. This statement holds without exception for all four of the esters. (3) That the accelerating action of sodium acetate decreases with increasing molecular weight of the ester. The reverse of this statement holds in the

¹ Unfortunately we could not obtain *n*-valeric or its sodium salt for our work.

main for the other salts. It is also noteworthy that whereas the hydrolysis of ethyl acetate is accelerated 60 per cent by sodium acetate the salts of the other three fatty acids retard its hydrolysis.

The following series shows the protecting action of these salts against the fluoride inhibition.

SERIES VII. Conditions identical with Series VI except that sodium fluoride was present in certain of the tubes as noted in the table. In all experiments in which the fluoride was used it acted in a concentration of 1 : 100,000.

Sodium salts.	Ethyl acetate.		Ethyl propionate.		Ethyl butyrate.		Ethyl valerate.	
	NaOH	Per cent	NaOH	Per cent	NaOH	Per cent	NaOH	Per cent
(Water).....	7.15		10.88		11.57		7.92	
Fluoride	0.58	-91.9	1.2	-89.0	2.4	-79.3	2.3	-71.0
Fluoride and acetate.....	2.48	-65.3	5.18	-52.4	9.65	-16.6	6.77	-14.5
Fluoride and propionate	2.63	-63.2	5.63	-48.3	9.9	-14.4	7.12	-10.1
Fluoride and butyrate.....	2.43	-66.0	5.43	-51.1	9.22	-20.3	6.87	-13.3
Fluoride and isovalerate.....	2.4	-66.4	5.58	-48.7	9.85	-14.9	6.95	-12.2

The first horizontal row of figures shows the hydrolysis of the esters in the absence of both the sodium salt of the fatty acid and the fluoride. The second row shows the hydrolysis in the presence of the fluoride alone. The remaining rows show the hydrolysis in the presence of both fluoride and fatty acid salt. This series shows (1) that for a given ester the sodium salts of all the fatty acids here studied have approximately the same protecting power against the inhibiting action of the fluoride, and (2) with increasing molecular weight of the esters up to ethyl butyrate, the protecting power of the salts of the fatty acids increases. The protecting power in the case of ethyl valerate is about the same as with ethyl butyrate. In regard to the mechanism of the antagonism between the fluoride and the salts of the fatty acids the experiments show conclusively that the accelerating action of the salts is entirely independent of their protecting action for the following reasons: (1) For a given ester we find that the accelerating action of the salts decreases with increasing mo-

molecular weight while the protective action remains the same. (2) With increasing molecular weight of the esters the accelerating action of sodium acetate diminishes while the protective action increases. (3) Sodium propionate, butyrate and isovalerate all inhibited the hydrolysis of ethyl acetate and yet they protected against the fluoride. Hence we conclude that the protecting and accelerating actions of the sodium salts of the fatty acids are probably independent phenomena. We do not care to hazard at present an explanation either of the accelerating or the protecting action of the salts. It is very interesting, however, that in order to completely neutralize the fluoride effect on the hydrolysis of ethyl butyrates in our experiments sodium butyrate had to be present in a molecular concentration 2100 times as great as the fluoride. The question arose whether sodium salts in general do not protect against the fluoride inhibition. We have found that sodium chloride which alone inhibits the hydrolysis of ethyl butyrate by the enzyme, has no effect on the inhibition by the fluoride. On the other hand, disodium phosphate accelerates the hydrolysis and protects against the fluoride inhibition. These facts would indicate that the salts of strong bases with weak acids in general probably exercise some protecting action against the fluoride inhibition. In many cases also these will be found to accelerate the hydrolysis of esters by the enzyme.

The results detailed above showing that the sodium salts of the fatty acids antagonize the inhibiting action of the fluoride offers a simple explanation of a fact brought out by Loevenhart and Peirce. It was found that the fluoride inhibition is considerably decreased by keeping the mixture neutral. This latter was accomplished by adding $\frac{N}{20}$ sodium hydroxide drop by drop as the acid produced by the hydrolysis developed. We now explain the decrease in the inhibition as due to the protecting action of the sodium butyrate produced by the addition of the alkali in these experiments.

The regularity seen in the action of the extract on the homologous series of esters, the regularity in the effect of sodium fluoride and the sodium salts of the fatty acids on the hydrolysis of these esters all prove that in the hydrolysis of these esters by liver extract only one enzyme is concerned.

The effect of sodium fluoride on the equilibrium reached in the action of lipase on ethyl butyrate. We have endeavored to determine whether sodium fluoride causes a shifting of the equilibrium point in the action of lipase on ethyl butyrate or whether it merely causes the reaction to proceed more slowly toward the same equilibrium point. The experiments were necessarily of long duration and the prolonged contact of the enzyme with the butyric acid produced during the reaction exercises a deleterious effect on the enzyme. This is such a disturbing factor that one could hardly hope to reach more than an approximate solution of this phase of the problem.

SERIES VIII. One cc. of a 10 per cent clear liver extract, 0.26 cc. of ethyl butyrate and 4 cc. of water or 3 cc. of water and 1 cc. of sodium fluoride were used in each experiment. The fluoride acted in a concentration of 1 : 100,000. Temperature, 38°.

Time, hours.	cc. of $\frac{N}{20}$ -NaOH required.		Inhibition, per cent.
	Water.	Fluoride.	
44	11.55	4.37	62
118	11.72	6.50	45
288	11.4	8.12	29
836	12.35	9.25	25

This series shows that in the absence of the fluoride the reaction proceeds very slowly after the first 44 hours. In other series we have observed the reaction approaches the equilibrium in 20 hours after which it proceeds very slowly indeed. In the presence of fluoride this is not the case. The reaction proceeds slowly but steadily for a much longer time. Even in experiments extending over a very long period of time, however, we have never seen the fluoride inhibition fall below 16 per cent when the fluoride acted in as great a concentration as 1 : 100,000.

In another series of experiments under conditions quite similar to Series VIII the enzyme was allowed to act for 21 hours when equilibrium had practically been reached. Then sodium fluoride was added and the reaction allowed to proceed for 52 hours when it was found that the fluoride exerted no influence on the course of the reaction. These facts all indicate that sodium fluoride in this reaction merely retards the progress of the reaction toward the equilibrium without affecting the position of the equilibrium

point. This is in keeping with the work of Loevenhart and Peirce and with the conclusion that the fluoride probably acts by retarding the formation or decomposition of some intermediate product formed in the hydrolysis of the ester by the enzyme. The facts that the fluoride can be dialysed from the enzymic solution leaving it with unimpaired activity and that the fluoride inhibition varies to such an extent when different esters are employed, both speak against the view that the fluoride simply reacts with the enzyme and decreases the active mass of the catalyser.¹

PART II.

A METHOD FOR THE DETECTION OF FLUORIDES IN FOOD PRODUCTS.

The method at present employed for the detection of small quantities of fluorides in food products all depend upon the etching of glass by the liberation of hydrofluoric acid after concentrating the fluoride. Woodman and Talbot² have recently improved and refined this method which in their hands is very delicate. They obtained a positive reaction when the fluoride was present in a concentration of 1 : 50,000,000.

It occurred to us that the remarkable effect of fluorides on the action of lipase might form the basis of a delicate method for the detection of fluorides. A large number of substances representing many different classes of compounds have been studied in reference to their effect on the action of lipase with the result that no other substances have been found which possess an inhibiting action comparable in extent with that shown by the fluorides. Many substances³ inhibit the action of lipase when present in sufficient concentration but at a dilution of 1 : 5000 the inhibition produced by most substances becomes negligible. In some cases, however, as with salicylic acid it is necessary to dilute to 1 : 20,000 when the inhibition becomes quite small and at 1 : 100,000 no inhibition is noted. On the other hand, the fluorides exert a powerful inhibiting action when present in a concentration of 1 : 1,000,000. This statement only applies to work with the 10 per cent clear liver extract and ethyl butyrate.

¹ See Loevenhart and Peirce, *loc. cit.*

² *Journ. Amer. Chem. Soc.*, xxviii, p. 1437, 1906.

³ See Kastle and Loevenhart: *Amer. Chem. Journ.*, xxiv, p. 491, 1900, and Loevenhart and Peirce, *loc. cit.*

When ethyl acetate is employed the inhibition is even greater. Thus when acting in a concentration of 1 : 5,000,000 sodium fluoride causes an inhibition of 50 per cent. In our work we have employed ethyl butyrate instead of the acetate, because the former is more readily hydrolysed by the liver extracts employed in this investigation. The technique depends somewhat on the nature of the mixture to be tested. In all cases, however, the following reagents are required:

- (1) Pure ethyl butyrate. This must be neutral.
- (2) $\frac{N}{20}$ sodium hydroxide.
- (3) Solution of litmus.
- (4) The enzyme solution as prepared by Loevenhart and Peirce, which is here described for the sake of completeness.

Ten grams of fresh pig liver such as can ordinarily be obtained in the market are ground with coarse white sand. About 40 cc. of water are then added and after grinding the mass with the water for a few minutes the mixture is strained through clean wet cloth and the residue again extracted with about 40 cc. of water and again strained through the same cloth. The extracts are combined and diluted to 100 cc. One cc. of toluene is then added and after stoppering and shaking thoroughly the mixture is placed in the thermostat at 35° to 40° from 24 to 36 hours when it will be found that on filtering through an ordinary folded filter a perfectly clear filtrate is obtained which varies in color from red to straw-color, according to the amount of blood present in the liver. It is best not to filter off more of the solution than is required for the work at hand as the turbid extract keeps for months if a small amount of toluene is kept in it. It should only remain in the incubator one day, however, as it keeps better at room temperature.¹

Our method is especially useful with foodstuffs containing no substance which inhibits the action of lipase. To this class belong meat and milk. In testing for fluorides in milk our method is as follows: Place in a test tube 1 cc. of the enzyme solution, 1 cc. of the milk to be tested, 3 cc. of water, 0.2 cc. of ethyl butyrate and 4 drops of toluene. Prepare a similar tube with 4 cc. of water and no milk or one containing 1 cc. of milk which is known to contain no fluoride and 3 cc. of water. Then stopper and shake the tubes well and place them in a thermostat at 35°

¹ The enzymic activity of these solutions vary considerably but we have never failed to obtain a preparation perfectly satisfactory for our purposes. The more active the preparation, however, the greater will be the effect of the fluoride.

to 40°. At the end of a few hours, preferably 14 to 18 hours, both tubes are removed, litmus added and titrated with $\frac{N}{20}$ sodium hydroxide. If the milk contains fluoride even in small quantities it will be noted that in the corresponding tube much less acid will have developed than in the tube which serves as a control. The inhibitions noted with milk containing various quantities of fluoride are shown in the following table:

SERIES IX. Duration of experiments, 18 hours. Temperature, 39°. The tubes were prepared as indicated above.

Amount of NaF per liter of milk.	$\frac{N}{20}$ NaOH required.	Inhibition, per cent.
<i>gm.</i>	<i>cc.</i>	
0.0	10.82	
0.2	2.52	76.7
0.02	5.95	45.0
0.01	6.82	37.0
0.005	8.02	25.9

Numerous specimens of fresh milk containing no preservative were examined. In most instances an acceleration was noted varying from 1 to about 20 per cent. In one instance, however, in which we had but a small amount of material and therefore could not convince ourselves by corroborative tests that it contained no fluoride, considerable inhibition was noted (35 per cent). More data on the effect of fresh milk, containing no preservative, on the action of the enzyme will have to be collected in order to determine the limits of the effect of such milk on the process.

It is interesting in this connection to note that the fluoride does not influence the rennin coagulation of milk when it is present in a concentration of 0.1 gram per liter.

We have not studied the effect of salicylic acid dissolved in milk on the action of the enzyme but aqueous solutions of it show the following inhibitions:

- 1.0 gram salicylic acid per liter, 45.0 per cent inhibition.
- 0.5 gram salicylic acid per liter, 10.6 per cent inhibition.
- 0.05 gram salicylic acid per liter, 0.6 per cent acceleration.

It is probable that when dissolved in milk similar values will be found. The last figure is within the limit of the experimental error. Sodium baborate, 1 gram per liter, and sodium benzoate, 10 grams per liter, in aqueous solution do not influence the action of lipase.

In our experiments with meat we used hamburger steak. Portions of 10 grams were treated with 1 cc. of a solution of sodium fluoride containing (1) 0.005, (2) 0.0005, (3) 0.0001 gram. Hence the meat contained per kilo in the three experiments, (1) 0.5, (2) 0.05, (3) 0.01 gram. These quantities were thoroughly incorporated with the meat which was then extracted with 10 cc. of water. The extracts were then boiled and about 7 cc. of filtrate expressed. Tubes were prepared containing 2 cc. of this filtrate, 1 cc. of enzyme solution, 2 cc. of water, 0.2 cc. ethyl butyrate and 4 drops of toluene. The inhibitions noted after 15 hours were as follows: (1) 93.7 per cent, (2) 70.1 per cent, (3) 41.8 per cent. Hence 0.01 gram of fluoride per kilo of meat can be readily detected and by a more careful extraction, evaporating the filtrate and using the total quantity there would be no difficulty in recognizing much smaller quantities. A similar aqueous extract prepared from 10 grams of meat which contained no preservative slightly accelerated the action of the enzyme.

The introduction of certain new methods of Effront in many breweries and distilleries makes it of some importance to recognize the presence of fluorides in beer and in brewery and distillery slops used for cattle feeding. In the case of both beer and grain substances are invariably present which inhibit the action of lipase even in the absence of any preservative. This difficulty can be overcome in the case of beer by diluting before making the test. The inhibiting action of beer which contains no preservative is shown in the following table:

SERIES X. Duration of experiments, 17 hours. Temperature, 37°. Each tube contained 1 cc. of enzyme solution, 0.2 cc. of ethyl butyrate and toluene. The total volume in each experiment was 5 cc. made up with water or beer as indicated in the table.

No. of experiment.	Water.	Beer.	cc. $\frac{N}{20}$ NaOH required.	Inhibition per cent.
1	4		8.7	
2		4	1.9	78.2
3	3	1	4.83	44.5
4	3	1*	0.8	90.8

* The same beer was used in all of these experiments, but in Experiment 4, 0.05g. of sodium fluoride per liter had been added to the beer.

These experiments show that when 1 cc of beer containing no preservative is used an inhibition of nearly 50 per cent is noted. It is true that if the beer contains 0.05 gram of fluoride per liter a much larger inhibition is noted (Experiment 4), but it is much more satisfactory to dilute 1 cc of the beer to be tested with 9 cc. of water and then employ 1 cc. of this dilution in each experiment in a total volume of 5 cc. We have tested three specimens of light beer obtained from different breweries and found that beer containing no fluoride gives under these conditions an inhibition of 1.8 to 5.7 per cent, whereas beer containing 0.1 gram per liter when similarly diluted causes an inhibition of from 56 to 60 per cent. In testing for smaller quantities of fluoride in beer we have evaporated 50 cc. in a crucible after adding 0.1 cc. of reagent strength sodium hydroxide and burned the residue (It is not desirable to burn off all the carbon.) The ash is then extracted with 5 cc. of water and 1 cc. of the filtrate is neutralized and employed in each test. With beer which contains no fluoride the filtrate will show no inhibition but if the beer contains 0.01 gram of fluoride per liter an inhibition of about 40 per cent is noted. This is certainly not the limit of delicacy of the method. By evaporating larger quantities of beer and using the entire filtrate from the ash 0.001 gram fluoride per liter could certainly be recognized.

We have also performed some experiments with canned tomatoes and with grain (rye). The former presents no difficulties, but grain extract powerfully inhibits the action of the enzyme. The ash of the grain also slightly inhibits the action of the enzyme (about 10 per cent) and our results when small amounts of fluoride were present have not been satisfactory thus far.

We present these data in their present shape, however, to indicate that the remarkable effect of the fluorides on the action of lipase can be made the basis of a simple and useful method for the detection of fluorides in food products, and in the hope that food chemists will improve the method from a technical point of view and show its limitations. The following precautions, however, must be here pointed out: (1) Any material which is to be tested must be made perfectly neutral before being mixed with the enzyme. (2) The initial acidity of the enzyme solution should be subtracted from the total acidity at the end of the

experiment. (3) The temperature of the thermostat should not be allowed to rise above 40°. (4) The enzyme should be allowed to act at least 4 hours and preferably over night (16 hours). (5) Many substances inhibit the action of lipase if present in sufficient concentration. Hence it is essential in testing for fluorides in a given material that some similar material which is known to contain no fluoride should be tested first or simultaneously in order to determine whether the material itself contains any inhibitory substance. If it does contain such a substance the difficulty may be overcome (a) by diluting to the point where the inhibitory action of the material becomes negligible in the absence of fluorides but which will persist if fluoride has been added to the foodstuff as a preservative, (b) by evaporating the material, incinerating the residue and extracting the ash with water. In the latter case the material should be made alkaline before evaporating and the attempt should not be made to burn off the carbon in the ash. It should not be heated to a higher temperature than is required to destroy most of the organic matter. Corroborative fluoride tests should be used in doubtful cases, but when no inhibition is noted it may be safely assumed that fluorides are not present in appreciable quantities.

SUMMARY.

In regard to the kinetics of the fluoride inhibition we have shown:

(1) That the inhibition decreases as we ascend in the fatty acid series of esters.

(2) That the sodium salts of the fatty acids, which alone accelerate the action of the enzyme, are capable of neutralizing the inhibitory effect of the fluorides when present in sufficient concentration. In order to completely neutralize the fluoride inhibition in our experiments sodium butyrate had to be present in a molecular concentration 2100 times as great as that of the fluoride. This neutralizing action is not a summation effect and is apparently independent of the accelerating action of these salts. The accelerating action of the salts decreases with increasing molecular weight of the salt; the protecting action of the sodium salts of acetic, propionic, butyric and valeric acids is the same for a given

ester; the protecting action increased with increasing molecular weight of the ester.

(3) The fluoride does not cause a shifting of the equilibrium point but merely retards the production of equilibrium.

The powerful inhibiting effect of fluorides on the action of lipase furnishes an interesting biochemical test for the presence of fluorides in food products and will doubtless prove of considerable value in the work of food inspection.

THE INFLUENCE OF HYDRAZINE UPON INTERMEDIARY METABOLISM IN THE DOG.¹

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Of the many pathological conditions experimentally evoked none is associated with greater evidences of noticeable abnormality than that induced by the subcutaneous administration of hydrazine. The researches of Borissow,² of Pohl³ and of Poduschka⁴ have demonstrated the relatively great toxicity of this compound and have defined the series of manifestations following its introduction into the body. With doses of 0.1 gram of hydrazine sulphate per kilo of body weight subcutaneously injected vomiting is observed which is succeeded by extreme restlessness. There is augmentation of the heart beat which later falls far below the normal, and respiratory difficulty is accompanied by general paralysis. At this stage a short period of coma usually ensues which terminates in death. The entire cycle of events is completed within a very few days. Coincident with the symptoms noted above is the appearance in the urine of variable quantities of protein and bile pigments together with appreciable amounts of allantoin crystals. The liver appears to suffer fatty metamorphoses, and autolysis of various tissues of such experimental animals leads to the presence of significant quantities of allantoin in the digestion mixtures.

The severe disturbances after hydrazine administration associated with the appearance in the urine of large quantities of

¹ This investigation was conducted with the aid of a grant from the Carnegie Institution of Washington to Prof. R. H. Chittenden at whose suggestion this work was carried out.

² Borissow: *Zeitschrift für physiologische Chemie*, xix, p. 499, 1894.

³ Pohl: *Archiv für experimentelle Pathologie und Pharmakologie*, xlviii, p. 367, 1902.

⁴ Poduschka: *Ibid.*, xlv, p. 59, 1900.

allantoin, the significance of which is as yet not satisfactorily explained, together with the pathological picture presented by the liver are apparently suggestive of broken links in the chain of normal intermediary processes. The assumption of a deranged condition of intermediary metabolism carries with it the suggestion of changed relationships in the urinary constituents. An investigation of these, especially of the partition of the various forms of nitrogen and sulphur, forms the basis of this paper.

Methods. The animals employed were full grown bitches which were catheterized daily. The feces were not analyzed. Total sulphur was determined by the method of Neumann,¹ the partition of the different forms of sulphur by the method of Folin.² Total nitrogen was estimated by the Kjeldahl-Gunning method, ammonia³ nitrogen, creatinin³ nitrogen, creatin⁴ nitrogen by the corresponding methods of Folin. Phosphorus was determined by titration with uranium nitrate. Urea nitrogen was estimated by a modification of the Mörner-Folin⁵ method. Inasmuch as allantoin appears to be decomposed in part by the Mörner-Folin process urea was extracted by an alcohol-ether mixture in which the ratio of alcohol to ether was 1 : 1 instead of 2 : 1 as suggested by Mörner. In the former solution urea is soluble while allantoin is insoluble according to observations made by Haskins⁶ which we have been able to confirm. By this modification allantoin nitrogen is not included with urea nitrogen. Purin nitrogen and that existing in the form of allantoin were estimated by a slight modification of the method employed by Poduschka.⁷ This method in detail is as follows:

To 50–100 cc. of urine is added basic lead acetate to complete precipitation. An aliquot portion of the filtrate is freed from excess of lead by means of hydrogen sulphide gas (instead of sodium sulphate as in the original method) and after driving off the hydrogen sulphide an aliquot portion of this filtrate is treated with a 10 per cent silver nitrate solution so long as a precipitate falls (20–30 cc.). This precipitate is thoroughly

¹ Neumann: *Zeitschrift für physiologische Chemie*, xliii, p. 37, 1904-05.

² Folin: *This Journal*, i, p. 131, 1906.

³ Folin: *American Journal of Physiology*, xiii, p. 45, 1905.

⁴ Folin: *Festschrift für Olaf Hammarsten*, Upsala, 1906.

⁵ Mörner: *Skandinavisches Archiv für Physiologic*, xiv, p. 297, 1903.

⁶ Haskins: *This Journal*, ii, p. 217, 1906.

⁷ Poduschka: *loc. cit.*

washed with water and the nitrogen contained therein, determined by Kjeldahl determination, is taken as the "purin nitrogen." An aliquot portion of the filtrate is treated with 1 per cent ammonium hydroxide solution to faint alkalinity (litmus) and then a large quantity (50-100 cc.) of 10 per cent solution of silver nitrate added. In the presence of allantoin a white flocculent precipitate forms which gradually falls to the bottom. This precipitate is washed free from ammonium hydroxide with 1 per cent sodium sulphate solution and nitrogen determined by the Kjeldahl process.

The original Poduschka method proved unsatisfactory in our hands as did all the other published methods for allantoin estimation.¹ The method as modified by us, that is, the substitution of hydrogen sulphide for sodium sulphate for the removal of excess of lead has proved most satisfactory. A solution of allantoin containing 0.056 gram nitrogen added to 100 cc. water gave 0.053 gram nitrogen in the silver precipitate. A second trial resulted in regaining 0.056 gram nitrogen when 0.058 gram nitrogen in the form of allantoin had been added to water. In many experiments with normal human urine we have never been able to detect allantoin; that is, in the final addition of silver no precipitation occurs. The same observation has held true with urine of dogs fed on a mixed diet (meat, cracker meal, lard). When allantoin has been added to normal human urine the amount regained has varied from 91.0 per cent to 99.0 per cent of the quantity added. Similar results have been obtained with dog's urine when the original urine was free from allantoin. To insure good results with this method, however, extreme care must be exercised in the addition of ammonia in which allantoin is readily soluble.

THE INFLUENCE OF STARVATION UPON THE DISTRIBUTION OF NITROGEN AND SULPHUR IN THE URINE OF THE DOG.

During hydrazine intoxication the animal refuses all food although water is drunk greedily. Consequently before any conclusions can be drawn regarding the partition of nitrogen and sulphur in the urine under hydrazine poisoning comparison should be made with the distribution of the above mentioned urinary

¹ Cf. Dakin: *This Journal*, iii, p. 57, 1907.

TABLE I.
Distribution of Nitrogen in the Urine During Fasting.
Dog 3.

Date.	Weight.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	Urea N.	NH ₃ N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	Purin N.	Allantoin N.	Undetermined N.	P.	Urea N.	NH ₃ N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	Purin N.	Allantoin N.	Undetermined N.
	gms.	cc.			gms.	gms.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.
1906																						
Nov. 27	13.1	130	1.046	acid	4.44	3.24	0.33	0.12	0.09	0.03	0.04	0.40	0.31	0.52	72.9	7.4	2.72	0.0	0.70	9.0	6.9	
28	12.8	125	1.038	"	3.26	2.78	0.13	0.08	0.08	0.00	0.05	0.15	0.07	0.34	85.2	3.9	2.42	0.0	0.15	4.9	2.1	
29	12.3	125	1.038	"	3.17	2.69	0.13	0.08	0.07	0.00	0.01	0.12	0.14	0.37	84.8	4.1	2.52	0.0	0.00	3.8	4.4	
30	12.2	150	1.026	"	3.29	2.90	0.18	0.11	0.09	0.02	0.01	0.11	0.00	0.33	88.1	5.5	3.32	0.0	0.60	3.3	0.0	
Dec. 1*	11.9	100	1.049	"	4.25	3.12	0.27	0.13	0.09	0.04	0.08	0.20	0.45	0.48	73.4	6.3	3.02	0.0	0.91	9.4	10.6	
2	11.9	90	1.030	"	2.67	1.73	0.28	0.07	0.05	0.02	0.01	0.20	0.38	0.24	64.8	10.4	2.61	0.0	0.70	4.7	14.2	
3	11.7	180	1.030	"	3.91	3.05	0.29	0.13	0.08	0.05	0.02	0.22	0.20	0.37	78.0	7.4	3.32	0.0	1.20	5.5	5.1	
4*	11.0	95	1.050	"	3.96	3.00	0.34	0.16	0.13	0.03	0.02	0.38	0.06	0.29	75.8	8.5	4.03	0.0	0.80	5.9	1.5	
5	11.0	170	1.020	"	3.86		0.15	0.07	0.05	0.02	0.04	0.16		0.28		3.9	1.81	3.05	1.0	4.0		
6	10.9	120	1.035	"	4.25	3.65	0.29	0.13	0.10	0.03	0.04	0.15	0.00	0.39	85.8	6.8	3.12	0.0	0.80	9.3	5.0	
7	10.9	90	1.045	"	3.17	2.40	0.25	0.08	0.05	0.03	0.03	0.26	0.15	0.31	75.7	7.9	2.51	0.0	0.90	9.8	2.4	
8	10.8	90	1.045	"	3.43	2.52	0.21	0.08	0.06	0.02	0.05	0.10	0.47	0.29	73.5	6.1	2.31	0.0	0.61	4.2	9.1	
9*	10.6	80	1.050	"	3.46	2.59	0.23	0.09	0.06	0.03	0.02	0.24	0.29	0.33	74.9	6.6	2.61	0.0	0.90	6.6	9.3	

* Allantoin crystals.

constituents which obtains during starvation. In the literature available at the commencement of these experiments we were unable to find records of such investigations on dogs.¹ Accordingly a bitch was allowed to fast, water being given freely, for a period of 14 days; and in the appended table are given the results obtained (see Table 1).

The changes apparent in the urinary nitrogen distribution during fasting when compared to that of a well fed animal² show (1) a slightly increased percentage of ammonia nitrogen and a correspondingly diminished urea percentage. This is in harmony with recently published experiments of Oesterberg and Wolf,³ although the latter investigators found the above relations changed to a much greater degree. In their experiments, however, the urine obtained was frequently alkaline in reaction which was never true in our own experiments. The possibility therefore suggests itself that the greatly increased ammonia output of their results may be explained in part as being of bacterial origin; that is, as a result of cystitis caused by catheterization. The investigations of Schöndorff⁴ have demonstrated that in hunger the percentage of urea nitrogen may fall to 75 per cent of the total nitrogen. (2) The total creatinin nitrogen does not bear a strict relation to the total nitrogen as the limits of variation are from 1.9-4.9 per cent of the total nitrogen, nor is the quantity excreted constant. The relatively large output of creatin at times is also of special interest although its significance at this time is obscure. Since there are no records of the output of creatin and creatinin during prolonged starvation in the dog, determined by recent methods, the following table is given showing the total creatinin, preformed creatinin, and creatin excretion. These figures are taken from the experiment represented in Table 1, where only the corresponding nitrogen has been given.

¹ Since the completion of this work a paper by Oesterberg and Wolf (*Biochemische Zeitschrift*, v, p. 304, 1907) has appeared giving such data but only for a few days period.

² Cf. Underhill and Closson: *This Journal*, ii, p. 117, 1906, and *American Journal of Physiology*, xvii, p. 42, 1906.

³ *Loc. cit.*

⁴ Schöndorff: *Archiv für die gesamte Physiologie*, cxvii, p. 257, 1907.

Total nitrogen	Total creatinin.	Preformed creatinin.	Creatin.
4.44	0.324	0.256	0.079
3.26	0.220	0.216	0.005
3.17	0.216	0.208	0.009
3.29	0.304	0.236	0.079
4.25	0.340	0.256	0.097
2.67	0.196	0.140	0.065
3.91	0.360	0.228	0.153
3.96	0.440	0.360	0.093
3.86	0.200	0.124	0.088
4.25	0.360	0.268	0.107
3.17	0.248	0.148	0.116
3.43	0.220	0.164	0.065
3.46	0.236	0.173	0.073

The most striking feature of the present experiment, however, is the constant presence of significant quantities of allantoin in the urine and the frequent separation in crystalline form. That the crystals obtained were allantoin was demonstrated by solubility, melting point (212° to 217°) and nitrogen determination. We have been unable to find any reference in the literature to the appearance of allantoin in the urine during starvation although numerous observations have been made concerning the derivation and elimination of this substance after feeding various compounds. From a survey of the literature¹ upon this subject it appears to us that like other nitrogenous substances of the body allantoin may have a twofold origin, one exogenous, the other endogenous. That allantoin is excreted in large quantities in the urine of dogs after ingestion of nuclear material is firmly established. The evidence of an endogenous source, however, has not hitherto been demonstrated. *Our finding of allantoin in the urine of starving dogs² shows without question that allantoin may have an endogenous origin.* Obviously the ultimate source of the endogenous allantoin may be identical with that of exogenous origin, namely, purin yielding material, although no fixed relation appears to exist between the purin output and allantoin excretion (see Table 2). In some of the published experiments

¹ For a résumé of the literature, see Mendel and White: *American Journal of Physiology*, xii, p. 85, 1904-05.

² A preliminary notice of the appearance of allantoin in the urine of the dog during inanition was published by one of us (U.) in the *Proceedings of the Society for Experimental Biology and Medicine*, iv, p. 144, 1907.

on allantoin formation fasting animals have been employed. It is therefore evident that such experiments will need revision before the conclusions drawn can be considered firmly established.

In other dogs the elimination of purin and allantoin nitrogen and the distribution of the different forms of sulphur during starvation has been followed. The results obtained are given in Table 2.

In inanition no fixed relationship appears to exist between the excretion of purins and allantoin. With respect to the partition of sulphur in the urine during starvation the only feature worthy of note is the relatively high proportion of "neutral" sulphur eliminated.

THE INFLUENCE OF HYDRAZINE POISONING UPON THE DISTRIBUTION OF URINARY NITROGEN AND SULPHUR.

Following a period of starvation a dog (same as in Table 1) was well fed for several weeks upon a mixed diet. A subcutaneous injection of hydrazine sulphate (0.1 gram per kilo body weight) was then administered after which the usual symptoms were observed. Corresponding to the starvation experiment the partition of nitrogen in the subsequent urinary output was determined. Inspection of the resulting data (Table 3) will show a lower output of ammonia and a somewhat higher urea nitrogen excretion than was observed in the same dog during inanition. The ratios of the purin nitrogen and of the creatinin nitrogen to the total nitrogen were somewhat higher than in the starvation period while the excretion of phosphorus was considerably greater. The proportion of preformed creatinin nitrogen was somewhat decreased with a corresponding increase in the creatin nitrogen. The relation of the creatinin and creatin is better seen in the appended table.

Total nitrogen.	Total creatinin.	Preformed creatinin.	Creatin.
3.43	0.280	0.180	0.116
5.54	0.540	0.320	0.255
8.83	0.752	0.360	0.455
3.60	0.340	0.118	0.258

TABLE 2.

*Excretion of Total Nitrogen, Purin Nitrogen, Allantoin Nitrogen and Sulphur in the Urine During Fasting.**Dog 4.*

Date.	Weight.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	Purin N.	Allantoin N.	Total S.	Inorganic S.	Ethereal S.	Neutral S.	Purin N.	Allantoin N.	Inorganic S.	Ethereal S.	Neutral S.
	k.	cc.			gms.	gm.	gm.	gm.	gm.	gm.	gm.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.
1907																
Jan. 21	10.0	100	1.020	acid	1.82	0.01	0.02	0.061	0.022	0.009	0.030	0.5	1.1	36.1	14.8	49.2
22	9.7	130	1.032	"	2.78	0.02	0.05	0.153	0.091	0.016	0.046	0.7	1.8	59.5	10.5	30.1
23	9.4	120	1.022	"	2.64	0.01	0.16	0.211	0.095	0.021	0.095	0.4	6.1	45.0	10.0	45.0
24	9.2	80	1.030	"	1.28		0.13	0.167	0.089	0.009	0.069		10.2	53.3	5.4	41.3
25	9.0	60	1.035	"	2.16	0.02	0.11	0.163	0.079	0.012	0.072	0.9	5.1	48.5	7.4	44.2
26	9.0	130	1.023	"	2.35	0.02	0.05	0.173	0.071	0.015	0.087	0.9	2.1	41.0	8.7	50.3
27	9.0	60	1.030	"	1.68	0.01	0.04	0.123	0.053	0.014	0.056	0.6	2.4	43.1	11.4	45.5

Dog 5.

Date.	Weight.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	Purin N.	Allantoin N.	Total S.	Inorganic S.	Ethereal S.	Neutral S.	Purin N.	Allantoin N.	Inorganic S.	Ethereal S.	Neutral S.
	k.	cc.			gms.	gm.	gm.	gm.	gm.	gm.	gm.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.
Feb. 4	12.8	177	1.064	acid	8.40	0.03	0.15	0.380	0.257	0.025	0.099	0.4	1.8	67.6	6.6	26.1
5		185	1.043	"	7.49	0.03		0.354	0.221	0.027	0.105	0.4		62.4	7.9	29.7
6		130	1.035	"	5.11	0.02	0.30	0.322	0.225	0.028	0.070	0.4	5.9	70.0	8.4	21.7
7		80	1.045	"	2.52	0.006	0.03	0.179	0.109	0.015	0.056	0.2	1.2	60.9	8.4	31.3
8		70	1.050	"	2.38	0.01	0.02	0.187	0.117	0.018	0.053	0.4	0.8	62.6	9.6	28.3
9		65	1.050	"	2.68	0.01	0.15	0.177	0.097	0.022	0.058	0.4	5.6	54.8	12.4	32.8
10		45	1.045	"	0.96	0.005	0.009	0.041	0.017	0.003	0.021	0.5	0.9	41.5	7.3	51.2

*Allantoin crystals.

TABLE 3.
Distribution of Nitrogen in the Urine During Hydrazine Poisoning.
 Dog S.

Date.	Weight.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	Urea N.	NH ₃ N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	Purin N.	Allantoin N.	Undetermined N.	P.	Urea N.	NH ₃ N.	Total creatinin N.	Preformed creatinin N.	Creatin N.	Purin N.	Allantoin N.	Undetermined N.	
1907	g.	cc.			gms.	gms.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.
Jan. 12	10.5	120	1.034	acid	3.43	2.76	0.26	0.10	0.07	0.03	0.05	0.26	0.0	0.30	80.5	7.5	2.9	2.0	0.9	1.4	7.5	0.0	
13	10.3	120	1.043	"	5.54	4.66	0.21	0.21	0.08	0.13	0.02	0.04	0.40	0.45	84.1	13.7	3.7	1.4	2.2	0.4	0.7	7.2	
14	10.3	220	1.042	"	8.83	6.98	0.32	0.28	0.13	0.15	0.07	0.60	0.58	0.72	79.0	3.6	3.2	1.5	1.7	0.8	6.8	6.6	
15*	10.3	90	1.040	"	3.60	2.66	0.10	0.13	0.04	0.09	0.08	0.15	0.48	0.31	73.9	2.8	3.6	1.1	2.5	2.2	4.2	13.3	

* Allantoin crystals.

The following figures were obtained from a male dog poisoned with hydrazine. The animal was not catheterized. Since urine was voided daily the quantity obtained was probably not far from that formed in the 24 hours.

Total nitrogen.	Total creatinin.	Preformed creatinin.	Creatin.
17.75	0.960	0.426	0.619
18.67	0.898	0.432	0.541
10.31	0.577	0.304	0.317
11.88	0.598	0.270	0.380
11.21	0.640	0.273	0.426
13.38	0.640	0.375	0.307
7.71	0.475	0.420	0.064

The relation of allantoin nitrogen to total nitrogen, however, remained almost constant during hydrazine poisoning and during starvation. This demonstrates that *a starving condition is the important factor in allantoin elimination and that hydrazine has no specific action in this respect* contrary to the statement of Poduschka that "Die Hydrazinvergiftung bildet durch die bei ihr constant eintretende Allantoin-ausscheidung ein Unicum in der toxicologischen Litteratur."¹

Dogs No. 4 and No. 5 after the period of starvation (see Table 2) were well fed for several weeks and then each received subcutaneously 0.1 gram hydrazine sulphate per kilo body weight. Unfortunately these animals did not survive sufficiently long to furnish data enough to draw definite conclusions regarding the elimination of purin and allantoin nitrogen and of the distribution of sulphur (see Table 4, Dogs 4 and 5). It is noticeable, however, that the proportion of "neutral" sulphur was significantly high when compared with that existing in the urine of the same animals during starvation. This finding was corroborated by analysis of the urine of another dog to which hydrazine had been administered in the same dosage (see Dog 6, Table 4).

After subcutaneous injections of hydrazine the urine of other dogs was tested for the presence of lactic, oxybutyric, and diacetic acids, acetone, and reducing bodies with negative outcome. Small quantities of kynurenic acid were present. In one animal there was an increased output (0.324 gram per day) of oxalic

¹ *Loc. cit.*

TABLE 4.
Excretion of Total N, Purin N, Allantoin N, and Sulphur in the Urine During Hydrazine Poisoning.
Dog 4.

Date.	Weight.	Volume.	Specific gravity.	Reaction to litmus.	Total N.	Purin N.	Allantoin N.	Total S.	Inorganic S.	Ethereal S.	Neutral S.	Purin N.	Allantoin N.	Inorganic S.	Ethereal S.	Neutral S.
	g.	cc.			gms.	gm.	gm.	gm.	gm.	gm.	gm.	p. ct.	p. ct.	p. ct.	p. ct.	p. ct.
1907																
Mar. 4	11.2	225	1.035	acid	3.43	0.02	0.20	0.487	0.250	0.041	0.195	0.6	5.8	51.3	8.4	40.0
5	11.0	65	1.030	"	1.37	0.02	0.13	0.176	0.043	0.018	0.115	1.5	9.5	24.4	10.2	65.3

Dog 5.

4	12.2	100	1.014	acid	0.85	0.01	trace	0.186	0.092	0.004	0.089	1.2		49.5	2.2	47.8
5		15		"	0.72			0.034			0.022					64.7

Dog 6.

13	9.1	260	1.030	acid	4.30	0.03	0.07	0.782	0.311	0.013	0.458	0.7	1.6	39.8	1.7	58.6
14		190	1.038	"	8.23	0.04	0.06	0.363	0.171	0.012	0.180	0.5	0.7	47.1	3.3	49.7
15		170	1.050	"	6.72	0.04	0.02	0.325	0.121	0.011	0.192	0.6	0.3	37.2	3.4	59.1
16		182	1.040	"	7.20		0.15	0.390	0.149	0.007	0.174		2.1	38.2	17.2	44.6
17																
18		320	1.034	"	5.35	0.04	0.08	0.481	0.218	0.013	0.250	0.7	1.5	45.3	2.7	52.1
19		440	1.028	"	6.67	0.07	0.15	0.497	0.239	0.007	0.251	1.0	2.2	48.1	1.4	50.5
20*		90	1.050	"	3.84			0.216	0.086	0.008	0.122			39.8	3.7	56.5

* Allantoin crystals.

acid, together with the separation of cystin in crystalline form. The substance was benzoylated and its behavior harmonized with that of cystin. Protein and bile pigments were generally present in the urine. The calcium content remained within normal limits.

A HISTOLOGICAL STUDY OF THE ACTION OF HYDRAZINE.

The influence of hydrazine upon the various organs and tissues of the body has received but scant attention. With the exception of the observations of Borissow who noted an increase in the size of the liver accompanied by hyperæmia, and the fatty changes of that organ as recorded by Pohl, nothing is known concerning the behavior of the drug upon cellular structures. This deficiency has been supplied through the courtesy of Prof. H. Gideon Wells of the University of Chicago, who has made a careful and systematic histological examination of organs and tissues obtained from dogs poisoned with hydrazine. He has summarized the results as follows: "Hydrazine seems to be a poison with an almost specific effect upon the cytoplasm of the parenchymatous cells of the liver, for when the poison is given subcutaneously this tissue alone shows evident structural alterations, although equal or greater amounts of hydrazine must reach other organs and tissues. It seems to have remarkably little effect upon other than hepatic cells, and does not cause any appreciable destruction of red corpuscles; slight hemorrhages are occasionally produced, but much less than by other poisons with a similar effect upon the liver. It attacks only the cytoplasm of the liver cells, never affecting the nucleus primarily, and causes a profound fatty metamorphosis of the type commonly referred to as "fatty degeneration." In this respect it resembles phosphorus, but differs in two important particulars: Hydrazine attacks first the cells in the center of the lobules, while phosphorus shows its first and most marked effects upon the peripheral cells; and secondly, phosphorus usually causes marked fatty changes in the myocardium, the kidneys, and indeed throughout the body, whereas the effects of hydrazine seem to be limited almost absolutely to the liver. The unknown poisons of acute yellow atrophy and eclampsia, and most of the bacterial poisons, attack first and

chiefly the nuclei of the liver cells, in contrast to the strictly cytoplasmic effects of hydrazine. Phosphorus also affects the nuclei more than does hydrazine.

As a poison for use in experimental studies of hepatic metabolism, hydrazine would seem to commend itself over phosphorus on account of its more selective action on the liver. In the maximum sublethal doses given for experimental purposes it will destroy fully as large an amount of liver tissue as will phosphorus and probably more; but there will always be left a considerable amount of liver tissue in a fair state of preservation, and presumably functionally competent, whichever poison is used. When recovery of the experimental animal is desired, the return of the liver to normal will probably be more rapid and more complete after hydrazine than after phosphorus."

The most striking feature of the action of hydrazine upon the animal body is the absence of abnormal relationships in the principal urinary constituents. Yet according to histological examination the liver is profoundly altered in structure and a large proportion of the cells are apparently inactive. The only inference that can be drawn from such evidence is that through the persistence of a small number of normally functioning liver cells this organ is enabled to maintain its intermediary metabolic processes in approximate equilibrium. This is in harmony with the recently published observations of Jackson and Pearce¹ upon the production of artificial liver necrosis by injections of hæmotoxic sera, and as they have aptly pointed out constitutes one of the best examples of the "factors of safety"² or protective adaptations in the animal body.

SUMMARY.

In starvation the dog excretes urine in which the proportion of ammonia nitrogen is slightly increased, and the urea nitrogen slightly decreased when compared to the relations that exist in the urine of an animal on a mixed diet. The creatinin excretion is variable, and there is a relatively large output of creatin. *Allantoin is a constant constituent of dog's urine during starvation.*

¹ Jackson and Pearce: *Journal of Experimental Medicine*, ix, p. 552, 1907.

² Meltzer: *Journal of the American Medical Association*, xlviii, p. 655, 1907.

In hydrazine intoxication the partition of urinary nitrogen and sulphur is only slightly different from that which obtains during inanition. *Hydrazine has no specific action in causing an elimination of allantoin.* It does, however, exert a specific influence upon the liver producing in the cytoplasm fatty metamorphoses of the type commonly referred to as "fatty degeneration."

Emphasis is laid upon the protective adaptation of the liver during hydrazine poisoning.

THE INFLUENCE OF POTASSIUM CYANIDE UPON PROTEID METABOLISM.¹

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It was shown by Claude Bernard² in 1857 that the venous blood of an animal poisoned with hydrocyanic acid takes on an arterial hue. Gaethgens³ in estimations of oxygen consumption during cyanide poisoning, was unable to find uniform variations from the normal which would explain the phenomenon. Geppert⁴ however by the use of improved methods succeeded in demonstrating very clearly that during and immediately after the convulsions produced by hydrocyanic acid the amount of carbon dioxide in the venous blood was very much less than in the venous blood of an animal undergoing convulsions as the result of electrical stimulation. In some cases it was less than in the venous blood of a resting animal. Geppert's conclusion that under the influence of cyanide the cells of the body lose to a certain extent their power of absorbing and utilizing oxygen has been universally accepted as explanatory of the phenomenon described by Bernard. Vernon⁵ has recently shown that the surviving kidney perfused with oxygenated Locke's solution under the influence of hydrocyanic acid takes up far smaller amounts of oxygen than in the absence of the cyanide.

¹ A report of these experiments was made before the Society for Experimental Biology and Medicine on October 16, 1907. *Proc. of the Soc. for Exp. Biol. and Med.*, v, p. 3, 1907.

² Bernard: *Leçons sur les effets des substances toxiques et médicamenteuses*. Paris, 1857, p. 193, et seq.

³ Gaethgens: Hoppe-Seyler's *Medicinisch-chemische Untersuchungen* Berlin, p. 325, 1866, cited from Geppert.

⁴ Geppert: *Zeitschr. f. klin. Med.*, xv, pp. 208 and 309, 1889.

⁵ Vernon: *Journ. of Physiol.*, xxxv, p. 70, 1906.

Investigations of the effects of cyanide upon processes of metabolism other than gaseous are few. Zillessen¹ showed that the sugar and lactic acid of the blood regularly increase under its influence. Herter and Wakeman² found that the application of solutions of potassium cyanide to the pancreas produced glycosuria.

The only observations concerning the effects of cyanide upon nitrogenous metabolism are those of Loewy. In a preliminary note³ he announced the fact that the nitrogen excretion in dogs may be increased by the administration of cyanide and stated his intention of subjecting this question to further study. In a later paper⁴ he described four experiments in which the total nitrogen and the caloric quotient of the urine was determined and in one of which the amount of nitrogen precipitable by naphthylisocyanate (amido-acids) was estimated. In three of his experiments an increase in total nitrogen occurred and in all the caloric quotient of the urine was increased. In the one experiment in which the estimation was made it was found that there was an increase in the amount of nitrogen precipitable by means of naphthylcyanate. He concluded that the most certain and typical result of cyanide poisoning is an increase in the caloric quotient of the urine for which the excretion of intermediary products of proteid cleavage is partly at least responsible.

Experiments made by one of us with Howland⁵ before the publication of either of Loewy's papers seemed to indicate that the transformation of indol to indican could be prevented by the action of potassium cyanide, and that under its influence indol might exhibit toxic properties not commonly attributed to it. Before a study of the changes in metabolism caused by indol and cyanide could be made it was necessary to determine more specifically than Loewy has done the alterations in metabolism which occur as the result of cyanide alone. For this reason and because of the great general interest connected with the study of a poison

¹ Zillessen: *Zeitschr. f. physiol. Chem.*, xv, p. 387, 1891.

² Herter and Wakeman: *Virchow's Archiv*, clxix, p. 489, 1902.

³ *Centralbl. f. Physiol.*, xix, p. 23, 1906.

⁴ *Biochem. Zeitschr.*, iii, p. 439, 1907.

⁵ *Proc. of the Soc. for Exp. Biol. and Med.*, iii, p. 71, 1906: *Archives of Pediatrics*, xxiv, p. 401, 1907.

which apparently exerts a specific effect upon oxidations we have carried out the two experiments described in this paper.

Methods. The animals used were dogs. In one experiment a uniform diet of fresh meat, cracker meal and lard was given and in the other no food was given throughout the experiment. The animals were kept in well-ventilated cages with movable trays for the collection of urine and feces. The urine of both dogs during the fore and after periods was collected entirely by catheterization. During periods of severe poisoning small amounts of urine, voided spontaneously, were immediately washed from the collecting tray with distilled water. The implements for catheterization were sterilized before using and the bladder was washed with 5 per cent boric acid solution each time. A few cc. of the boric acid solution were left in the bladder after each collection of urine. There was never any admixture of urine with feces or vomitus and no urine was lost.

The total urine of the 24 hours was combined with the washings of the bladder (and on days of poisoning with washings of collecting tray) and made up to known volume with water.

Total nitrogen was estimated by the Kjeldahl method, urea, ammonia, creatin and creatinin by the methods of Folin.¹ Total sulphur, total sulphate, and ethereal sulphate were determined gravimetrically as barium sulphate, observing all of the precautions suggested by Folin.² Phosphoric acid was determined by titration with uranium nitrate, potassium ferrocyanide being the indicator. Acidity was not determined on account of the use of boric acid in washing the bladder. Each portion of urine, tested by litmus, was found to be acid.

The total nitrogen of feces and vomitus was determined in Experiment I by the Kjeldahl method. The material was prepared for analysis by grinding with water, rubbing through a fine sieve and making up the homogeneous paste so obtained to known volume with water. The close agreement of duplicate

¹ *Amer. Journ. of Physiol.*, xiii, pp. 45, et seq., 1905. *Festschrift für Hammarsten*, 1906.

² *This Journal*, i, p. 131, 1906. For the use of the Duboscq colorimeter we are indebted to the courtesy of Dr. Beebe and Dr. Shaffer of the Loomis Laboratory of the Cornell University Medical College, where the determinations of creatin and creatinin were carried out.

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determinations made on aliquot portions indicate the accuracy of this way of preparing the material for analysis.

DESCRIPTION OF EXPERIMENTS.

EXPERIMENT I. Well-nourished, fox terrier bitch; weight, 11.10 kilos. Had been kept in the laboratory for two years and was thoroughly accustomed to surroundings and to catheterization.

*Normal period, August 20, 21 and 22.*¹ Catheterized and bladder washed with boric acid solution daily at 8:30 a. m. Weighed and fed immediately after. Food consisted of 152 grams of lean, hashed beef (N = 3.56 per cent), 50 grams of cracker meal (N = 1.79 per cent), 15 grams of lard (N = 0.14 per cent) and 350 cc. of water. The total nitrogen content of this diet was 6.318 grams. Catheterized and bladder washed at 5 p. m. and 11 p. m. daily. Water (200 cc.) given at 6 p. m.

Potassium cyanide period, August 23, 24 and 25. August 23. Catheterized at 8:30 a. m. and immediately weighed and fed. At 2:30 p. m. injected subcutaneously 1.4 cc. of freshly prepared, 2 per cent solution of potassium cyanide (= 2.5 mg. per kilo). No symptoms followed. At 8 p. m. catheterized and injected subcutaneously 1.4 cc. of 2 per cent potassium cyanide (= 2.5 mg. per kilo). Slight nausea resulted but no vomiting. Defecated three times, the last portion containing much fluid. Catheterized at 10:30 p. m.

August 24. Catheterized 8:30 a. m., weighed and fed immediately. At 2:15 p. m., after catheterization, injected 1.65 cc. 2 per cent potassium cyanide (3.0 mg. per kilo). No symptoms appeared in the following two hours. At 4:25 p. m. and at 5:25 p. m. injected 0.825 cc. of 2 per cent potassium cyanide (2×1.5 mg. per kilo). No symptoms had appeared at 7:15 p. m. and 1.1 cc. of 2 per cent potassium cyanide (2 mg. per kilo) was injected. The same dose was again given at 8:45 p. m. and half the dose at 9:15 and again at 9:45 p. m. This was followed by defecation (twice, the second being fluid), by vomiting (once) after symptoms of nausea, and by some incoördination of muscular movements. These symptoms had disappeared at 11:30 p. m. No feces, vomitus or urine passed during the night.

August 25. Catheterized at 8:30 a. m., weighed and fed immediately after. At 2:45 p. m. injected subcutaneously 1.65 cc. 2 per cent potassium cyanide (3 mg. per kilo) and 0.55 cc. (1 mg. per kilo) at 3:25. A small amount of material was vomited and there were several attempts to defecate. At 3:55 and again at 4:40 p. m. 0.55 cc. (1 mg. per kilo) of potassium cyanide solution was injected. Salivation, twitching of muscles of face and slight incoördination of muscular movements resulted. At 5:12 p. m. injected 0.55 cc. of potassium cyanide solution (1 mg. per kilo). Severe tonic-clonic convulsions occurred at 5:32 and 5:50 p. m. During this interval and continuing till 6:20 there was complete prostration.

¹ Dates represent the days on which the 24 hour periods ended.

Rectal temperature sank to 36.5° C. At 6:30 recovery had apparently begun and 0.55 cc. potassium cyanide solution was injected. The same amount again given at 7 p. m. Severe convulsions each lasting for one-half to one minute began at 7:08 and continued till 9:30 p. m. There were about 40 of these convulsions. During the last half-hour of this period the animal was kept warm with cloths and electric lamps. Recovery, beginning at 9:30 was rapid and at 10:15 she walked about in a normal fashion; 300 cc. of water given at 10.30. Catheterized at 10.45.

After period, August 26, 27 and 28. Catheterized and fed as in normal period. No symptoms could be observed which were attributable to the poisoning except moderate general depression on the first day of this period.

EXPERIMENT II. Mongrel bitch, fairly well nourished; weight, 10.24 kilos. Not excitable and offered no resistance to manipulation incident to catheterization. No food was given from August 21 until the experiment was ended. Water was given *ad libitum*.

Fore period, August 24 and 25. Catheterized daily at 8:45 a. m. Bladder washed with boric acid solution. Weighed immediately. Catheterized at 11 p. m.

Potassium cyanide period, August 26. Catheterized at 8:45 a. m. At 9:10 a. m. injected subcutaneously 1.49 cc. of freshly prepared, 2 per cent potassium cyanide solution (3 mg. per kilo). Vomited once. At 10:15, 0.75 cc. potassium cyanide solution given. Vomiting, defecation, muscular incoördination and a period of mild convulsions lasting from 10:50 to 11:20 a. m. resulted. At 1:50, 2:25 and 2:50 p. m. 0.75 cc. potassium cyanide injected. At 3:35, 0.37 cc. was given. Vomiting followed by convulsions from 3:45 to 4:15 p. m. occurred. At 4:40, 0.37 cc. 2 per cent potassium cyanide injected. Marked depression resulted. At 7:40 p. m. injected 1 cc. 2 per cent potassium cyanide (2 mg. per kilo) and one-half this dose at 8:10 and 8:40. Vomiting and a few mild convulsions resulted. At 9:18 injected 0.25 cc. and at 9:35, 1 cc. 2 per cent potassium cyanide. Severe convulsions from 9:20 until 11:30 p. m. resulted. These ceased at 11:30 when she was completely prostrated, making shrill cries and ineffectual movements of the legs. Not observed later than 12 p. m. The following morning behavior was normal save for moderate general depression. There were in all during this day about 35 convulsions. Dyspnoea was very marked during and after these seizures.

After period, August 27 and 28. Catheterized as during fore period. No abnormalities of any sort save slight depression on the 27th. At expiration of experiment ate greedily.

The analytical results obtained are given in the tables on pp. 184, 185 and 186. Other data which have not been included in the tables are as follows: No albumin was eliminated in the urine of either dog, at any time during the experiments. Sugar was detected in the urine of August 26 in Experiment II. No acetone could be detected by Legal's test at any time.

TABLE I.
Excretion of Nitrogen in Experiment I. Total Nitrogen of Daily Diet = 6.318 gms.

Date, viii.	Body weight, kilos.	Vol. of urine, cc.	Total N of urine, gms.	Urea N, gms.	Ammonia N, gm.	Urea + ammonia N, gms.	Creatin N, gm.	Creatinin N, gm.	Total creatinin N, gm.	Undeter- mined N, gm.	Nitrogen in feces, gm.
1907.											
20	11.25	482	5.583	4.784	0.318	5.102	0.164	0.137	0.301	0.180	1.117
21	11.25	405	5.609	4.847	0.255	5.102	0.155	0.137	0.292	0.215	
22	11.22	470	5.967	5.050	0.312	5.362	0.160	0.137	0.297	0.308	
23	11.10	545	6.312	5.398	0.312	5.710	0.147	0.146	0.293	0.309	1.838
24*	10.89	309	6.221	5.469	0.193	5.662	0.189	0.140	0.329	0.230	
25†	10.63	397	6.833	5.750	0.250	6.000	0.244	0.101	0.345	0.488	
26	10.71	450	6.547	5.636	0.338	5.974	0.264	0.135	0.399	0.174	0.902
27	10.80	410	6.000	5.219	0.247	5.466	0.148	0.136	0.284	0.250	
28	10.87	355	5.447	4.790	0.234	5.024	0.095	0.126	0.221	0.202	

* Nitrogen of vomitus = 0.082 gm. † Nitrogen of vomitus = 0.301 gm.

TABLE II.
Excretion of Nitrogen in Experiment II.

Date, viii.	Body weight, kilos.	Vol. of urine, cc.	Total N of urine, gms.	Urea N, gms.	Ammonia N, gm.	Urea + ammonia N, gms.	Creatin N, gm.	Creatinin N, gm.	Total creatinin N, gm.	Undeter- mined N, gm.
1907.										
24	9.95	162	2.961	2.449	0.141	2.590	0.067	0.105	0.172	0.199
25	9.90	95	3.059	2.612	0.122	2.734	0.043	0.108	0.151	0.174
26	9.12	110	3.592	3.014	0.148	3.162	0.126	0.061	0.187	0.243
27	9.30	130	4.718	4.037	0.206	4.243	0.104	0.100	0.204	0.271
28	9.15	185	3.397	2.882	0.229	3.111	0.041	0.096	0.137	0.149

On examination of the urines for lactic acid¹ crystals having the form of zinc lactate were found in Experiment I on August 23 and 24 (combined), August 26, 27 and 28; in Experiment II on August 24 and 25 (combined), August 26 and 27. Indications of its presence in the urine of the other days were absent. No

TABLE III.

Excretion of Sulphur and Phosphorus in Experiment I.

Date viii.	Total sulphur.	Total su'phate S.	Inorganic sulphate S.	Ethereal sulphate S.	Neutral sulphur.	P ₂ O ₅ .
1907	gm.	gm.	gm.	gm.	gm.	gm.
20	0.3432	0.2038	0.1796	0.0242	0.1434	0.745
21	0.3488	0.2142	0.1928	0.0214	0.1346	0.780
22	0.3916	0.2527	0.2230	0.0297	0.1389	0.840
23	0.4511	0.3172	0.2829	0.0343	0.1339	1.190
24	0.2972	0.1703	0.1538	0.0165	0.1269	0.750
25	0.3911	0.1908	0.1862	0.0046	0.2003	1.260
26	0.3752	0.1802	0.1505	0.0297	0.1950	0.738
27	0.3411	0.1900	0.1703	0.0197	0.1511	0.780
28	0.2991	0.1933	0.1752	0.0181	0.1066	0.757

TABLE IV.

Excretion of Sulphur and Phosphorus in Experiment II.

Date viii.	Total sulphur.	Total sulphate S.	Inorganic sulphate.	Ethereal sulphate.	Neutral sulphur.	P ₂ O ₅ .
1907	gm.	gm.	gm.	gm.	gm.	gm.
24	0.1840	0.1109	0.0900	0.0209	0.0731	0.405
25	0.1774	0.0961	0.0911	0.0051	0.0813	0.395
26	0.2414	0.1161	0.1112	0.0049	0.1253	1.140
27	0.1692	0.0549	0.0472	0.0077	0.1142	0.223
28	0.1977	0.1032	0.1021	0.0010	0.0945	0.291

more than one-tenth of the total volume of urine of any day could be used for the determination and the amounts of crystals were too small to admit of analysis.

Uric acid determinations according to Folin² were made in

¹ Hoppe-Seyler-Thierfelder: *Handbuch d. chem. Analyse*, Berlin, p. 449, 1903.

² *Amer. Journ. of Physiol.*, xiii, p. 49, 1905.

TABLE V.
Distribution of Nitrogen and Sulphur in the Urine in Experiment I.

Date viii, 1907.	IN PER CENT OF TOTAL NITROGEN.						IN PER CENT OF TOTAL SULPHUR.				
	Urea N.	Ammonia N.	Urea + ammonia N.	Creatin N.	Creatinin N.	Total creatinin N.	Undeter- mined N.	Total sulphate S.	Inorganic sulphate S	Ethereal sulphate S.	Neutral sulphur.
20	85.69	5.70	91.39	2.94	2.45	5.39	3.22	59.38	52.33	7.05	40.62
21	86.42	4.55	90.97	2.76	2.44	5.20	3.83	61.41	55.27	6.14	38.59
22	84.63	5.23	89.86	2.68	2.30	4.98	5.16	64.53	56.93	7.60	35.47
23	85.52	4.94	90.46	2.33	2.31	4.64	4.90	70.31	62.71	7.60	29.69
24	87.91	3.10	91.01	3.04	2.25	5.29	3.70	57.30	51.74	5.56	42.70
25	84.15	3.66	87.81	3.57	1.48	5.05	7.14	48.78	47.60	1.18	51.22
26	86.09	5.16	91.25	4.03	2.06	6.09	2.66	48.02	40.11	7.91	51.98
27	86.98	4.12	91.10	2.47	2.26	4.73	4.17	55.70	49.92	5.78	44.30
28	87.94	4.30	92.24	1.74	2.31	4.05	3.71	64.62	58.57	6.05	35.38

TABLE VI.
Distribution of Nitrogen and Sulphur in the Urine in Experiment II.

Date viii, 1907.	IN PER CENT OF TOTAL NITROGEN.						IN PER CENT OF TOTAL SULPHUR.			
	Urea N.	Ammonia N.	Urea + ammonia N.	Creatin N.	Creatinin N.	Undeter- mined N.	Total sulphate S.	Inorganic sulphate S.	Ethereal sulphate S.	Neutral sulphur.
24	82.71	4.76	87.47	2.26	3.55	6.72	60.02	49.02	11.00	39.98
25	85.39	3.99	89.38	1.40	3.53	5.69	54.17	51.34	2.83	45.83
26	83.91	4.12	88.03	3.51	1.70	6.76	48.09	46.06	2.03	51.91
27	85.57	4.37	89.94	2.20	2.12	5.74	32.44	27.89	4.55	67.56
28	84.84	6.74	91.58	1.21	2.83	4.38	52.20	51.64	0.56	47.80

Experiment I on the urines of August 20 and 21 (combined) and August 25; in Experiment II on the urines of August 24 and 25 (combined) and August 26. The results obtained were as follows:

Experiment I.

August 20 and 21 (average).....	26.25 mg. uric acid
" 25	56.25 " " "

Experiment II.

August 24 and 25 (average)	16.9 " " "
" 26	59.5 " " "

SUMMARY OF RESULTS.

Total nitrogen. In both experiments an increase is seen during the poisoning, the maximum increase in Experiment I amounting to about 15 per cent and in Experiment II to about 20 per cent. On the day following poisoning this increase persisted, being about 10 per cent in Experiment I; in Experiment II the increase on the day following poisoning was more marked being over 50 per cent. In both animals on the second day after poisoning it had dropped to practically normal.

Urea and ammonia nitrogen. The curve of urea excretion follows that of total nitrogen very closely in each experiment. This is also true of the sum of urea and ammonia nitrogen. There is no increase of ammonia nitrogen in either dog during the poisoning. The figures for ammonia nitrogen in the after period of Experiment I are closely comparable to those of the normal period; in Experiment II there is an increase in the after period.

In Experiment I the nitrogen in the form of urea and ammonia expressed as percentage of total nitrogen is slightly lower on the day of severest poisoning (August 25); we consider this change to be within the limits of normal variation. In Experiment II, the percentages of urea and ammonia nitrogen are uniform throughout.

Creatin and creatinin. The nitrogen eliminated as creatinin increases slightly in Experiment I on the days of mild poisoning (August 23 and 24), but was markedly diminished on the day of severest poisoning. In Experiment II, a marked diminution occurred on the day of poisoning (August 26). In both experiments the excretion was normal throughout the after period.

The creatin nitrogen increased decidedly on the day of severest poisoning in Experiment I and on the poison day of Experiment II. In both experiments this change persisted for one day following. The increase in creatin nitrogen was greater than the decrease in creatinin nitrogen, and hence the total creatinin nitrogen in both animals increased on the days of severe poisoning and on the day after. Expressed as percentages of total nitrogen, an increase in creatin nitrogen and a decrease in creatinin nitrogen occurred as a result of severe poisoning in both animals. The percentage of total creatinin increased in Experiment I only on the day following severest poisoning, while in Experiment II there is rather a tendency to decrease throughout.

Undetermined nitrogen. In both experiments an increase in undetermined nitrogen took place on the days of severe poisoning. In Experiment I this increase was followed on the next day by a marked decrease; in Experiment II the increase persisted for one day.

In the percentage values of undetermined nitrogen there is no departure from normal limits except in Experiment I on the day of severest poisoning when the percentage is high and on the following day when it is low.

Sulphur. In Experiment I an increase in total sulphur amounting to about 20 per cent occurred on the first day of poisoning (August 23), an actual fall on the second day when the poisoning was slightly more intense and a rise to normal on the third day of poisoning (severest).

In Experiment II an increase of 33 per cent was observed on the one day of poisoning. Unlike the total nitrogen the total sulphur excretion was normal in amount on the day after poisoning.

Comparison of the figures for total sulphate sulphur with those for neutral sulphur shows that in Experiment I a rise in sulphate sulphur with no change in neutral sulphur occurred on the first day of (mild) poisoning; while a rise in neutral sulphur with no change in sulphate sulphur occurred on the day of severest poisoning. In Experiment II, on the one day of poisoning a rise of both sulphate and neutral sulphur was observed, the increase in the latter being twice as great as in the former. The increase in neutral sulphur excretion, unlike the sulphate sulphur excretion, continued during the first day of the after periods.

Expressed as percentages of total sulphur, the sulphate sulphur increased on the day of mildest poisoning in Experiment I and decreased on the days of severe poisoning and on the first day of the after period in both experiments. The variations in the percentage values of the neutral are of course the reverse of these.

Phosphoric acid. The variations in the excretion of phosphoric acid are similar to those of total sulphur.

DISCUSSION OF RESULTS.

The most striking changes which have occurred as the result of poisoning with cyanide are those connected with the excretion of total nitrogen, creatin and creatinin and with the distribution of sulphur.

In considering the causes of the increase in total nitrogen so clearly evident in these experiments, a number of possible factors present themselves. The more important ones seem to us to be these: Cell dyspnœa produced by the action of cyanide which has been demonstrated by the researches of Geppert; increase in muscular work caused both by labored respiration and by convulsions; some more specific action of the poison which is not the result of either of these.

The action of dyspnœa produced by mechanical obstruction to respiration, by rarefaction of the inspired air or by respiration of carbon monoxide has been the subject of many investigations.¹ Most of them agree in showing that dyspnœa produced by any of these means may cause an increased elimination of nitrogen which persists for some time (two or more days) after the respiratory interference has been removed. Voit² has contended that the increased nitrogen excretion, representing as it does, increased tissue destruction may not properly be attributed to diminution in the amount of oxygen inspired but is rather to be ascribed to the excessive muscular movements attendant upon the dyspnœic respiration. Although it is known that excessive muscular work under certain conditions of nutrition may cause increased nitrogen excretion, it is our opinion that Voit has not attributed

¹ A summary of these investigations with references is given by Voit, see below.

² Voit: *Zeitschr. f. Biol.*, xlix, p. 1, 1907; see also Fränkel, *ibid.*, 1, p. 163, 1907.

sufficient importance to certain experiments in which this element was lacking. Fränkel and Geppert¹ have described experiments on dogs in which the animals were made to breathe rarefied air under conditions which prevented abnormally high accumulation of carbon dioxide in the blood. The dyspnœa produced by the reduction of the pressure of the air breathed by the animal from normal to 230 millimeters was not excessive. In one experiment² in which the reduced pressure was maintained for over seven hours there was not a sign of increased depth or frequency of respiration, the animal remaining perfectly quiet throughout the entire period. The characteristic increase of urinary nitrogen on the two following days occurred. This experiment with others which gave identical results seems to prove conclusively that lessened air supply alone is capable of increasing the nitrogen eliminated.

We must assume on the basis of the amounts of potassium cyanide administered to our dogs and the symptoms exhibited that in these animals during a period of at least two hours (on the third day of poisoning in Experiment I and on the single day of poisoning in Experiment II) a condition was produced comparable to that in Geppert's³ animals in which cell dyspnœa was demonstrated. Since this condition seems to be strictly comparable with that produced in the air-rarefaction experiments of Fränkel and Geppert, we must conclude that the cell dyspnœa produced by the cyanide was in part at least responsible for the increase in total nitrogen of the urine. It can not be denied however that the muscular movements involved in the convulsions and extreme respiratory exertions probably contributed to this effect.

That potassium cyanide may produce an increase in nitrogen excretion for reasons other than the two which we have considered is indicated by the results obtained in Experiment I on the first day of poisoning. On this day the only symptoms of poisoning observed were a slight purgation and very slight and transitory signs of nausea. Geppert's analyses of the gases of

¹ *Ueber d. Wirkungen d. verdünnten Luft auf d. Organismus*, Berlin, pp. 95, et seq., 1883.

² The second experiment of *Versuchsreihe II*.

³ *Zeitschr. f. klin. Med.*, xv, p. 309, 1889.

the blood give no indication of diminished capacity on the part of the cells to utilize oxygen at this stage of poisoning. On the contrary there may be an actual increase in the amount of carbon dioxide formed under these conditions. Obviously also there was no excess of muscular work in our animal at this time. We must believe then that in this one experiment an amount of potassium cyanide too small to produce either respiratory dyspnoea or cell dyspnoea increased the total nitrogen excretion. Hence we must conclude that we are not justified in attributing the greater increase on days of severe poisoning exclusively or even mainly to any one of the three factors which are under consideration.

The fact that the urea excretion in our animals followed so closely the elimination of total nitrogen shows that no appreciable interference in the urea-forming processes has been produced. In other words, cell-dyspnoea of fairly great intensity does not prevent urea-formation. This affords additional evidence in favor of the view that the process of urea-formation is not oxidative in nature.¹

The ammonia throughout our experiments showed no variations beyond normal limits either of absolute or percentage values. If anything there is a slight percentage decrease on the day of severest poisoning. We must conclude that no acidosis was produced.

A distinct increase in preformed creatinin was observed on the first day—that of very mild poisoning—in Dog 1. With both dogs, on the day of severe poisoning a more distinct decrease occurred. On the day following the severe poisoning the excretion of this substance is normal in amount in both dogs. We have given reasons for supposing that the increase in total nitrogen on the first day of poisoning in Dog 1 was due neither to muscular work nor cell-dyspnoea. On the day of severe poisoning when the latter factors were active, a still greater rise in total nitrogen was found. Since muscular work has either no influence upon creatinin excretion or increases it² it is tempting

¹ Cf. Kossel and Dakin: *Zeitschr. f. physiol. Chem.*, xlii, p. 181, 1904; Folin: *Amer. Journ. of Physiol.*, xiii, p. 124, 1905.

² Van Hoogenhuyze and Verploegh: *Zeitschr. f. physiol. Chem.*, xlvi, p. 415, 1906.

to suppose that the property of the poison which increased total nitrogen on the first day can be differentiated from its power of interfering with cell-respiration by the fact that the former causes an increase and the latter a decrease in the excretion of preformed creatinin. Furthermore, the marked increase in total nitrogen which occurred on the day of severe poisoning in each dog attended by a decrease in creatinin might indicate that an endogenous or tissue metabolism of which creatinin is not an index may exist. The possibility must be borne in mind however that cyanide may have interfered with some of the intermediate steps in creatinin formation of whose nature we are ignorant and that it might have been possible to detect the elimination of some precursor of creatinin had we possessed the requisite knowledge.

The adherents of the older views regarding the physiological relationship between creatin and creatinin would say that we already had evidence of increased excretion of such a precursor of creatinin, namely, creatin. The work of Folin¹ and Klercker² has however thrown serious doubt upon the existence of such a relationship and indicates that in man the metabolism of each is independent. Although the most obvious explanation of the variations in creatin and creatinin which we have observed would be that an increase of creatin formation together with a decrease in its transformation to creatinin resulted from the action of cyanide, we cannot definitely state that this is the case until it has been shown that the observations of Folin and Klercker do not hold true of the metabolism of creatin in the dog. In both of our experiments the increase in creatin under the influence of severe cyanide poisoning is greater than the decrease in creatinin. On the day following severe poisoning in both animals the creatinin excretion had returned to normal while creatin was yet excessive. These facts indicate that a completely compensating relationship between the two is absent. Since the present state of our knowledge of creatin metabolism is very unsettled, no detailed explanation of the variations in its excretion which we have observed has been attempted. It is worthy of note that considerable creatin was eliminated by the fasting dog throughout the total period of observation (5 days).

¹ *Festschrift für Olof Hammarsten*, 1906.

² *Biochem. Zeitschr.*, iii, p. 45, 1907.

Our experiments have not given uniform results in respect to changes in undetermined nitrogen. In the starving dog (Experiment II), while there is an absolute increase on the day of poisoning, it is not great enough to produce any increase in the percentage value. In fact the figures for undetermined nitrogen in this animal cannot be said to vary beyond normal limits throughout the whole period of observation.

In Experiment I, on the day of severe poisoning, there is an increase of undetermined nitrogen great enough to increase the percentage value by 75 per cent. The corresponding decrease in urea and ammonia nitrogen on this day might indicate that the excess of undetermined nitrogen was derived from substances which normally would have given rise to urea and ammonia. As we have stated, we believe the variations in the latter forms of nitrogen on this day to be hardly beyond normal limits of variation. They certainly seem to be too slight to show any marked interference with processes of deamidization or any significant increase in autolysis which might lead to the excretion of amido-acids. In the starving dog (Experiment II) in which one would expect the production of autolysis by any means to be more apparent, there was no change in the relative excretion of undetermined nitrogen, and in the fed dog (Experiment I) the change observed on the day of severest poisoning had entirely disappeared on the following day. If processes of autolysis had been inaugurated by the large amounts of cyanide we should have expected them to be evident through a longer period.

The only direct evidence regarding the cause of an increase in undetermined nitrogen in our experiment is to be found in the determinations of uric acid. Although the amounts eliminated even on the days of severe poisoning are too small to be regarded as explanatory of a variation in undetermined nitrogen, they might be considered as indicating a decided disturbance in purin metabolism which might have come more clearly to light had we been able to determine the purin bases and allantoin. One would expect to find that the cyanides exert a rather specific effect upon processes of purin metabolism in which oxidation plays a part.

In the absence of more direct evidence, and in spite of Loewy's results it seems improbable that in our experiments a significant excretion of amido-acids took place.

In neither of our experiments did the total sulphur excretion follow closely the excretion of total nitrogen. In Experiment I the only marked increase above normal was on the first day of poisoning; the total nitrogen was highest on the third day. In Experiment II the greatest increase in total sulphur occurred on the day of poisoning while the total nitrogen attained its greatest height on the day following.

The rise in total sulphur excretion which occurred in Experiment I on the first day of poisoning and the fall on the next succeeding day were due chiefly to variations in the sulphate sulphur for the neutral sulphur remained nearly constant on these two days. On the day of severest poisoning in Experiment I the figure for sulphate sulphur was below normal while in Experiment II a slight rise occurred. In other words, mild poisoning with potassium cyanide produced a greater increase in oxidized sulphur excretion than did severe poisoning.

The results are different in the case of neutral sulphur. In Experiment I it showed a tendency to decrease during the two days of mild poisoning, though the variations from normal are slight. On the day of severest poisoning on the other hand there was an increase to over 40 per cent above the average normal value, the oxidized sulphur remaining, as has been said, below normal. In Experiment II on the day of poisoning the neutral sulphur increased by more than 60 per cent while the oxidized sulphur increased by only 12 per cent. The increase in neutral sulphur continued in both experiments during the following day.

The change in the distribution of the sulphur is most strikingly shown by a comparison of the figures for the third normal and third poison day of Experiment I. While the total amounts of sulphur eliminated are the same (.3916 and .3911 gram) the percentage of sulphur eliminated in the oxidized state is 64.53 on the normal day and 48.78 on the poison day. Obviously in this instance the distribution of the sulphur does not depend upon the absolute amount of sulphur present. It is probable that the principle in the chemistry of metabolism formulated by Folin¹ that the distribution of sulphur depends upon the absolute amount present can not have such a wide application as to include

¹ *Amer. Journ. of Physiol.*, xiii, p. 73, 1905.

results obtained when the metabolism is of a distinctly pathological type.

Although from the data which we have obtained it is impossible to say that on the days of severe poisoning with cyanide the neutral sulphur increases at the expense of sulphur which otherwise would have been oxidized, we believe that this explanation is probably the chief one.

Our results on the sulphur distribution are in general similar to those obtained by Reale and Boeri¹ by means of mechanically produced dyspnoea.

The small absolute amounts of ethereal sulphate and the fact that cyanide causes purgation make it difficult to draw any definite conclusions, from our figures relating to this form of sulphate.

¹ *Wiener med. Wochenschr.*, 1895, pp. 1064 and 1198.

THE PURGATIVE INEFFICIENCY OF THE SALINE CATHARTICS WHEN INJECTED SUBCUTANEOUSLY OR INTRAVENOUSLY.

A REPLY TO BANCROFT.

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In an interesting article, published a few years ago, J. B. MacCallum stated that "*all those salts which act as purgatives when introduced into the stomach or intestine have the same action when injected subcutaneously or intravenously.*"¹ His conclusion was of interest for the following reasons: in the first place, it was in opposition to the results obtained by most other investigators,² and secondly, it enriched therapeutic measures, for it frequently might be desirable to administer purgatives by another way than the mouth and the small doses employed seemed to permit this.

As a reinvestigation seemed desirable the therapeutically important salts were tested regarding their purgative effect when injected subcutaneously or intravenously. In these experiments the same animals (rabbits) and the same doses which MacCallum had utilized were employed. The salts used were magnesium sulphate, sodium sulphate, sodium phosphate and sodium citrate. The results obtained³ did not confirm the generalization of MacCallum quoted above; moreover, not only did no purgation occur but in some cases a definite constipation seemed to be produced. Tables were given in the published report which illustrated the uselessness of the salts mentioned as purgative agents when injected into the subcutaneous tissue or the bloodstream. The

¹ MacCallum: *Amer. Journ. of Physiol.*, x, p. 102, 1903. Italics not mine.

² For references, see Auer: *Amer. Journ. of Physiol.*, xvii, p. 15, 1906.

³ Auer: *ibid.*, p. 25.

results seemed so clear and convincing that the question could be regarded as settled in favor of the older investigators. Recently, however, Bancroft¹ has come forward with both a critique of my work and a claim that "MacCallum's results have been confirmed in every respect"² by him.

Before entering upon a discussion of Bancroft's paper, I may state at once that he not only utterly fails to explain my results but that his own work, when analyzed, also fails to corroborate MacCallum.

Bancroft's Criticism.

Bancroft gives the following explanations³ for my failure to obtain MacCallum's results:

(1) That I used only the "milder purgatives" employed by MacCallum.

(2) That I kept no adequate controls.

In regard to the first objection it must be pointed out that naturally only those salts were tested regarding which there was a doubt, and those salts were the ones employed in human therapeutics: magnesium sulphate, sodium sulphate, sodium phosphate and sodium citrate. Barium chloride was not used, because no one doubts its purgative effect when injected subcutaneously or intravenously. Moreover, I fail to understand why my use of the saline cathartics in the dosage employed by MacCallum constitutes a reason for my failure to obtain MacCallum's results: a two to sixfold increase in the fecal output.⁴

The second objection is even more ill-founded, and is best answered by a glance at a résumé of some of my published tables.

In the 36 experiments published in the former paper,⁵ 21 showed a *zero output one hour after the injection*; 27 showed not more than *two grams of feces after one hour*; and 25 passed not more than *four grams of feces during five to six hours after the injections*.

¹ Bancroft: *This Journal*, iii, p. 191, 1907.

² *Ibid.*, p. 193.

³ *Ibid.*, p. 210.

⁴ MacCallum: *loc. cit.*, p. 103.

⁵ Auer: *op. cit.*

February 15.	Total feces in 5 hours.	February 16.	Total feces in 6 hours.
Rabbit 1.....	0 grams	Rabbit 5.....	0
Rabbit 2.....	3 grams	Rabbit 6.....	0
Rabbit 3.....	1 gram	Rabbit 7.....	4 grams
Rabbit 4.....	1 gram	Rabbit 8.....	0
Control 1.....	1 gram	15 cc. 25 per cent solution of sodium sulphate subcutaneously.	
Control 2.....	14 grams		
15 cc. $\frac{M}{8}$ sodium sulphate subcutaneously.			
March 3.	Total feces in 6 hours.	March 9.	Total feces in 6 hours.
Rabbit 13.....	0	Rabbit 13.....	0
Rabbit 14.....	13 grams	Rabbit 14.....	1 pellet
Rabbit 15.....	1 gram	Rabbit 15.....	0
Rabbit 16.....	2 grams	Rabbit 16.....	4 grams
15 cc. $\frac{M}{8}$ sodium phosphate subcutaneously.		2 cc. $\frac{M}{8}$ sodium sulphate intravenously.	
March 13.	Total Feces in 4 hours.	March 8.	Total feces in 5 hours.
Rabbit 13.....	0	Rabbit 9.....	0
Rabbit 14.....	0	Rabbit 10.....	0
Rabbit 15.....	0	Rabbit 11.....	0
Rabbit 16.....	7 grams	Rabbit 12.....	0
2 cc. of a 25 per cent solution sodium sulphate intravenously.		2 cc. $\frac{M}{8}$ Sodium phosphate intravenously.	

These tables show that the output of feces in most experiments was practically zero for four to six hours after the injection of sodium sulphate and sodium phosphate, subcutaneously or intravenously; the doses and concentrations employed were those used by MacCallum, with the exception of the two series where a 25 per cent solution of sodium sulphate was used. Now what could controls show with regard to these experiments? If the controls also passed no feces in the same length of time as the experimental rabbits this could only mean that the injected solutions had no purgative effect; if the controls passed more than the injected rabbits, this again could only mean that the injected solutions constipated the animals. *This fact, that a minimal or zero fecal output of the injected animals during the experimental time required no controls, was early realized, though*

not early enough to avoid considerable work studying the amount of feces passed by normal rabbits when confined in separate cages for some time.¹ Yet Bancroft says that adequate controls would have shown the increase in the amount of feces which I was probably getting.² Even a cursory glance at the published tables should have shown him that his explanation demanded the excretion of practically less than no feces from those "adequate controls" in the majority of experiments. It is therefore clear that Bancroft's "explanation" of my results is unfounded.

Bancroft's Experiments.

Bancroft (p. 193) divides his experiments into two groups:

(1) "Those which simply repeated MacCallum's experiments, small doses being used."

(2) "Those in which the largest possible doses were used, in order to obtain fluid feces."

A consideration of the experiments quoted shows that Bancroft did not repeat MacCallum's work. Bancroft habitually uses 30 cc. of an $\frac{M}{6}$ solution, while MacCallum used only 10 to 15 cc. of an $\frac{M}{8}$ solution;³ the latter calls this amount a "fairly large quantity," while Bancroft calls two to three times that quantity of a *stronger* concentration, a "small dose." Moreover Bancroft's repetition confines itself to the subcutaneous injection of sodium citrate only. No intravenous injections of the ordinary salines, using MacCallum's doses (1 to 2 cc. of an $\frac{M}{8}$ solution), are given in his paper.

Let us now consider what Bancroft's experiments which form the basis of his Table I really show. He used four rabbits of approximately the same size and weight and confined them in separate cages. "In one pair the same rabbit was always the control, in the other pair *one animal was control one day and the experimental animal the next.*"⁴ This means that two rabbits received 30 cc. of an $\frac{M}{6}$ sodium citrate solution subcutaneously

¹ Auer: *loc. cit.*, p. 15. In the course of this paper a few tables showing the hourly output of feces passed by a number of normal rabbits when kept in separate cages will be given.

² Bancroft: *loc. cit.*, p. 210.

³ MacCallum: *loc. cit.*, p. 103.

⁴ Bancroft: *loc. cit.*, p. 193-194. Italics mine.

every other day; one rabbit never received any injection at all; and the fourth rabbit received 30 cc. of the solution every single day. He also changed the diet of the rabbits, which had been fed on hay, grain and vegetables, to only carrots and water. The feces of all the rabbits were then weighed after certain intervals, and Bancroft figures out, using the statistical method, that the injected animals passed during the first three to five hours after the injection, 23 times more feces than the "controls."¹ *He overlooks an important point however in making this calculation. On the same page he mentions that "a period of constipation follows the purgation due to the citrate."*² This in order to explain why the rabbits passed feces only on alternate days. *But these constipated rabbits were used as controls in the experiment.* If Bancroft thought these animals constipated, why did he use them as adequate controls? This error explains his astonishing result. The only control in the series was Rabbit 3 which received no injection whatsoever, and of this rabbit Bancroft³ states that it had "the diarrhoea," though the output to me seems perfectly normal and comparable to many which I have obtained. *It is clearly evident therefore that all of Bancroft's calculations based on Table I are worthless, and they will not be considered further here.*

This table however furnishes some other information; it shows what an extremely modest output of feces Bancroft is willing to consider a purgation. In his Table I the record of 16 injections of sodium citrate into three rabbits in eight days is given. The total yield during the first three to five hours after the injection was 109.3 grams, the average "purgation" therefore amounted to about 7 grams in three to five hours. Normal rabbits frequently pass as much as that and more in one to two hours (see Tables A, B, C, D); the importance of this striking variability in the fecal output of normal rabbits will be pointed out a little more fully when the question of controls is taken up. Under these conditions, is it justifiable to consider such a meager amount of scybala the result of a purgation especially as *normal*

¹ *Ibid.*, p. 194.

² *Ibid.*, p. 194.

³ Bancroft: *loc. cit.*, p. 196.

rabbits often pass much more in a shorter time? I do not think so. By purgation something perfectly obvious is meant, something which does not require statistical research for identification. As a definition the one I gave in a former paper should, I think, still hold: "By purgation is here understood the passage of soft and unformed feces in amounts exceeding that which normal animals might conceivably pass;"¹ for apparently normal animals at times pass some soft and unformed feces in small amounts. With this definition, which embodies the usual conception of a purgation, there need hardly be any doubt about the purgative effect of any injection.

Bancroft's statement² that the amount of feces eliminated in a certain time, *irrespective of consistency*, must be considered in determining whether or not "purgation" has occurred, is admissible only if at least four control rabbits are kept. The reason is this: normal rabbits do not pass equal amounts of feces during the same time; certain rabbits may pass nothing during the day while during the night a normal quantity is eliminated; or most of the scybala are passed during the day, and only few during the night; or approximately equal amounts are passed during the day and night. The same rabbit may show the above variation at different times. There are still other variations, an apparently normal rabbit may be constipated for no definite reason for a day or more, at irregular intervals. Most of these fluctuations are brought out in Tables A, B, C, D. Bancroft's tables also show many of these variations very well;³ (see Table I, p. 195, Rabbit 3; Table II, p. 197, Rabbits 3 and 4; Table III, p. 200). These large variations among normal rabbits make a number of controls imperative, if moderate accuracy is desired; otherwise the results would surely deceive, for a "purgation" in Bancroft's sense can easily be figured out from the normal output of five rabbits (Table C). If Rabbit VIII had received an injection, for instance, the results would have simulated a well-marked "pur-

¹ Auer: *loc. cit.*, p. 17.

² Bancroft: *loc. cit.*, p. 192.

³ Bancroft also recognizes fluctuations in the daily output (p. 196), but seems to think that these variations affect the controls in the same way at the same time.

gation." On the other hand, if the ordinary criteria of a purgation are accepted, no such mistake could be made.

Bancroft's Table II, p. 197, furnishes the proof that sodium citrate injected subcutaneously in double and treble the quantity employed by MacCallum, not considering even the stronger concentration, does *not* purge. A résumé of the table is here reproduced.¹

Day.	Hour.	No 2. 1501 grams subcutaneous.		No. 3. 1697 grams control.	No. 4. 1621 grams. control.
		Dose.	Feces.	Feces.	Feces.
		cc.	grams.	grams.	grams.
1	First 1 hour.....	30	13.8	8.3	2.4
	" 3 hours		21.6	14.9	5.9
2	First 1 hour.....	30	0.0	2.3	4.3
	" 4½ hours.....		8.7	23.8	5.5
3	First 1 hour.....	30	8.8	7.8	8.8
	" 4 hours		18.2	24.8	18.1
4	First 1 hour.....	30	10.0	2.1	13.4
	" 4 hours		21.0	16.1	32.9
<i>Totals:</i>					
	1st hour.....		32.6	20.5	28.9
	3-4½ hours.....		69.5	79.6	62.4

Animals were fed hay, grain and water.

It will be seen that in every experiment except the first, one or both of the controls at all times passed at least as much as the injected animals. *Three out of four experiments therefore were against the view Bancroft set out to prove, that sodium citrate purges when given subcutaneously.* Yet in spite of this markedly negative result, Bancroft ventures to make this misleading statement, though it is literally correct in a way: "It is only by comparing the totals that it can be seen that in spite of the fact that the controls are larger animals" (controls are 1-200 grams heavier) "and pass more feces, yet during the first hour after the administration of the citrate the experimental animals passed the greater amount of feces."² Now this holds true of the first hour *total* only, and not of the individual experiments. The method of comparing totals and averages, which Bancroft uses extensively in his paper,

¹ Accentuation of some of the figures due to the writer.

² Bancroft: *loc. cit.*, p. 198.

is methodologically wrong and gives false impressions when applied to a few experiments; it is safe to use only when large numbers of data are at hand. On the whole however the experiments did not seem satisfactory to Bancroft; he says that as the daily output of the rabbits increased greatly, due to their diet of hay, grain and water, "it is not surprising that the effects of small doses of sodium citrate might be hard to detect."¹ *Bancroft therefore admits that under some perfectly normal conditions rabbits show no purgation after the subcutaneous injection of "small doses" of sodium citrate; for a "purgation" which is "difficult to detect" has not even an academic interest.* Regarding the doses, let me point out again that Bancroft's "small doses" are two to three times larger than those employed by MacCallum, moreover they are also of a higher concentration.

Sodium citrate is the only ordinary saline which Bancroft employed in doses short of enormous, and even this salt he only used subcutaneously; none was given intravenously in moderate doses. Barium chloride, whose purgative action is doubted by no one, was tested; in this connection it seems necessary to state that barium chloride is not to be classed among what are ordinarily called the saline purgatives, and no other writers, as far as I am aware, so classes it.

Some New Experiments.

Although the above analysis of Bancroft's experiments and arguments shows clearly the incorrectness of his claim, a new series of experiments was carried out in which only sodium citrate in $\frac{M}{8}$ solution was injected, special attention being paid to the controls and to the hourly output. The following tables also illustrate the fluctuations which normal rabbits show and to which perhaps sufficient attention has already been called. A few additional words may, however, be permissible. The series of tables appended represent only a few of those obtained from nine rabbits observed in two divisions for twenty-four days. The rabbits in each division were approximately of the same size, and averaged 1400 and 2400 grams. At the end of the period of observation they had all gained in weight, with the

exception of Rabbits I and IX. They were all fed the same amount and kind of food at the same time, and were confined in separate cages in the same room.

TABLE A.

July 29.

Time.	I. White and gray rabbit, male.	II. White and black, female	III. Black, male.	IV. Gray, female.
8 to 9 a. m.	1 pellet	0 feces	0 feces	0 feces
12 m.	3 grams	2 grams	13 grams partly soft	12 grams
1.30 p. m.	2 pellets	7 "	11 grams	10 "
2.30 "	1 pellet	4 "	5 "	5 "
3.30 "	2 pellets	2 "	4 "	5 "
4.30 "	5 grams	2 "	9 "	5 "
5.30 "	5 "	4 "	2 "	1 "
to 8 a. m., July 30	5 "	12 "	9 "	27 "
Total, 24 hrs.	19 "	33 "	53 "	65 "

8 a. m., fed wet oats
4 p. m., fed cabbage leaves. } Equal amounts to each rabbit.
Average weight, 1,400 grams

TABLE B.

August 20.

Rabbit.	V. White, female	VI. Gray, female.	VII. Gray, male.	VIII. Gray, male.	IX. Gray, male.
9 to 10 a. m.	0 feces	0 feces	0 feces	0 feces	0 feces
11 "	0	0	4 grams	some soft 2 gram	0
12 m.	0	2 pellets	2	0	0
1 p. m.	0	0	1	5 gram	2 pellets
2 "	0	0	3	2.5	0
3 "	0	0	0.4	5.5	0.6
4 "	0	0	(2 pellets) 0.4	0.7	0
5 "	0	0	1.5	1 pellet	0.1
6 "	0	1 pellet	0	0	2
to 9 a. m., Aug. 21	18.5	22	29	21.5	21.5
Total, 24 hrs.	18.5	22	40	37	24

9 a. m., fed wet oats.
5 p. m. cabbage and carrot greens. } Same amount to each rabbit.
Average weight, 2,400 grams.

TABLE C.
August 21.

Rabbit.	V.	VI.	VII.	VIII.	IX.
9 to 10 a. m.	0 feces	1.5 gram	2 gram	some soft 12 gram	1.5 gram
11 "	0	1.5	0.5	1.5	0 irregular shape
1 p. m.	0	0	0.5 some soft	12	1
2 "	0	1 pellet	2 soft	9	1
3 "	0	1	1.5 irregular shape	0	1
4 "	1.5 gram	0	2	0	0.5
5 "	0	0	1.5	0	0.6
6 "	0	0	2	1	2.5
to 9 a. m., Aug. 22	26	33	1.5	21.5	13
Total, 24 hrs.	27.5	37	13.5	57	21

9 a. m., fed wet oats.

5 p. m. cabbage and carrot greens.

TABLE D.
August 22.

At 11.15 a. m. Rabbits V and VII received 15 cc. $\frac{M}{8}$ sodium citrate solution subcutaneously, lumbar region.

Rabbits.	V.	VI control.	VII.	VIII control.	IX control.
9 to 10 a. m.	0 feces	0	0	3.5 grms.	0
11 "	0	0	1 pellet (1 pellet)	9	0
12 "	0	0	0.1	5.5	0
1 p. m.	0	0	0	4	0
2 "	5	0 (1 pellet)	1	2 (1 pellet)	0
3 "	3	0.1	1.5	0.1	0
4 "	2	0.9	0	0	0
5 "	2.5	1	0	0	1
6 "	0	0	1 soft	0	3.5
to 9 a. m., Aug. 23	13	4	3.5	11	9
Total, 24 hrs.	25.5	6	7	35	13.5

9 a. m., wet oats.

5 p. m., cabbage and lettuce.

On August 22 (Table D) Rabbits V and VII received subcutaneously 15 cc. of an $\frac{M}{8}$ sodium citrate solution at 11:15 a.m. Nothing resembling a purgation resulted: Rabbit VII passed 1.2 grams and Rabbit V 5 grams during three hours after the injection. *Control VIII passed 11.5 grams in the same length of time;* Controls VI and IX passed practically nothing being in a stage of physiological constipation just as the injected Rabbit VII was. This experiment illustrates well the danger of too few control rabbits; if control Rabbit VIII had been absent a meager "purgation" would have resulted on paper, especially if averaged.

To sum up the above considerations of Bancroft's first group of experiments:

(1) Bancroft did not repeat MacCallum's work, for he used much larger doses.

(2) His results do not show that the injections bring on any purgation.

(3) A new series of experiments confirmed completely my former results, at least as far as sodium citrate is concerned. The fallacy is pointed out in using only one or two control rabbits in experiments of this nature.

Bancroft's Second Group of Experiments.

The second group, excluding barium chloride, deals entirely with the effects of *toxic* and *lethal* doses of some saline purgatives. These experiments are not relevant to the question at issue, for it is obvious that the purgative injections must be harmless, just as harmless as when administered *per os*. This was fully realized by MacCallum, for otherwise he certainly would not have advised that "the subcutaneous or intravenous administration of some of the salts, especially sodium citrate, might be safely resorted to"¹ in human beings. In spite of this, these experiments will be considered, for they throw light on Bancroft's methods and reasoning. For instance, in order to obtain fluid feces by the intravenous method, he injected usually about 400 cc. of an $\frac{M}{6}$ sodium sulphate solution through the marginal

¹ MacCallum: *loc. cit.*, p. 108-109.

ear vein.¹ This quantity corresponds approximately to four times the entire quantity of blood which an average rabbit possesses. After flooding the vascular system with such an amount of liquid it is not surprising that he obtained fluid feces from the animals which did not succumb. But it is surprising that Bancroft considers this an indication that sodium sulphate purges when injected intravenously, for 0.6 per cent sodium chloride solutions when injected in large amounts into the bloodstream cause a greatly increased fluidity of the gut contents, as was well shown in the famous experiments of Cohnheim and Lichtheim in 1877.² These authors state that under the conditions mentioned "the lumen of the gut always contains a very considerable quantity of a thin more or less fecal fluid." It is evident that Bancroft's experiment proves nothing regarding a distinctive purgative effect of sodium sulphate *per se* when injected with enormous quantities of liquid into the circulatory system. That Bancroft did not arrive at this conclusion is probably due to his controls. It seems that he considered perfectly normal rabbits controls for these experiments, for nowhere does he mention that his controls received an equal infusion of physiological saline solution. But a normal rabbit, surely cannot be considered an adequate control for an experiment in which the vascular system of the experimental animal is so swamped with fluid that "œdematous, jelly-like masses in the vicinity of the kidney"³ are found. If proper controls had been kept; that is, controls which received an equal quantity of normal physiological saline solution intravenously, Bancroft probably would have been able to corroborate Cohnheim and Lichtheim's findings and therefore would have hesitated to ascribe a purgative action to sodium sulphate *per se* when injected intravenously in this manner.

The doses employed for subcutaneous injections were enormous: for instance, sodium sulphate was injected in amounts

¹ Bancroft: *loc. cit.*, p. 201, 206.

² Cohnheim and Lichtheim: Virchow's *Archiv*, lxix, p. 121, 1877.

³ Bancroft: *loc. cit.*, p. 204. These œdematous masses Bancroft considers "direct evidence" that the urine carried with it much of the sodium sulphate injected.

varying from 60 to 110 cc. of an $\frac{M}{2}$ (16 per cent!) solution.¹ This would correspond for a 70 kilogram individual, to 3900 cc. or to 624 grams of the crystalline sodium sulphate. No wonder that "the injected animals were seriously injured and often killed"² by these doses. Most practitioners will probably hesitate to give over a pound of sodium sulphate subcutaneously in order to obtain a purgation.

When sodium citrate was given subcutaneously, almost invariably lethal doses had to be given in the attempt to obtain fluid feces, for in only one case did a rabbit survive a dose greater than 100 cc. of an $\frac{M}{6}$ solution,³ and we may be certain that no smaller dose had the desired effect. On postmortem examination of these rabbits Bancroft found fluid feces in the "large intestine" and argues that they would have been passed had the animals lived a little longer; he does not mention the condition of the intestinal walls.

This second group of experiments shows strikingly that Bancroft was not satisfied with the purgative effect of rational doses of salines when injected subcutaneously or intravenously, and really was forced to use these tremendous doses in order to obtain some approximation to the result obtained when the salines were given by mouth.

Magnesium sulphate.—In an appendix to his article, Bancroft extends his criticism to a paper by Dr. Meltzer and the writer, which deals with the relation of magnesium sulphate and chloride to intestinal peristalsis.

This and other researches had been undertaken on the basis of a hypothesis that all magnesium salts exert an inhibitory effect. In agreement with this hypothesis it was found that both magnesium sulphate and chloride, when injected subcutaneously or intravenously, did not cause intestinal peristalsis or purgation. Moreover, these injections directly inhibited existing peristalsis. Now MacCallum states that magnesium sulphate when injected subcutaneously or intravenously causes purgation,⁴ and explains this action by the statement that intestinal peri-

¹ Bancroft: *loc. cit.*, p. 200.

² Bancroft: *loc. cit.*, p. 199.

³ Bancroft: *ibid.*

MacCallum: *loc. cit.*, p. 108.

stalsis is stimulated. In commenting on this difference between MacCallum and ourselves with regard to magnesium sulphate, Bancroft speaks of a "misconception" (p. 208) of Dr. Meltzer and the writer in believing that MacCallum ascribes the purgative action of this salt to the magnesium which it contains; he also offers a theory of its action, according to which the SO_4 ion stimulates and the Mg ion inhibits, thus producing peristalsis and purgation. The difference however is not one of theory but of fact; what we stated were facts based on numerous observations. On the basis of still more numerous observations we may repeat that magnesium sulphate as well as magnesium chloride do not purge when injected subcutaneously or intravenously.

Bancroft apparently made no observations of his own with magnesium sulphate; he merely offered a theory. If he wishes to oppose his theory to our facts, we shall not argue with him. We wish however to disagree emphatically with an important part of his theory, that "calcium inhibits more strongly than magnesium" (p. 209). The reasons for this disagreement will be given in communications shortly to be published.¹

Bancroft's Disagreements with MacCallum.

As Bancroft's avowed purpose² is to corroborate the results of MacCallum's experiments, it may be not out of place to call attention to some discrepancies in the unanalyzed testimony itself.

To illustrate:

(1) MacCallum found that the subcutaneous injections of small doses of salines *constantly* increases two to sixfold the amount of feces eliminated during the following time interval.³

Bancroft's data for Table II force him to admit that under

¹At the meeting of the American Physiological Society in Chicago, December, 1907, and also at the December meeting of the Society of Experimental Biology and Medicine, experiments were demonstrated which conclusively showed the antagonistic action of calcium to the inhibitory action of magnesium. Moreover, calcium hastens and magnesium delays the onset of rigor mortis (*Journ. of Exp. Med.*, x, p. 45, 1908).

²Bancroft: *loc. cit.*, p. 191.

³MacCallum: *loc. cit.*, p. 103.

some perfectly normal conditions the purgative effect of those injections could not be detected.¹

(2) MacCallum states that "a much larger dose" is required to produce the same effect when the salines are introduced into the stomach or intestine than when injected subcutaneously or intravenously.²

Bancroft, on the other hand, devotes much space to an explanation why sodium citrate and sulphate are "so much more effective" in producing fluid feces when given *per os* than when injected subcutaneously or intravenously.³

(3) MacCallum noticed that the feces were sometimes semi-fluid after the subcutaneous injection of small doses of salines, and calls particular attention to this.⁴

Bancroft could not obtain this increased fluidity;⁵ moreover, he states definitely that "the action of small doses of weak purgatives [the salines] is to increase only the *amount* of feces eliminated * * *"⁶ differentiating clearly in the preceding sentences between amount and consistency.

These differences alone should have prevented Bancroft from stating, without any modifying clause, that "MacCallum's results have been confirmed in every respect," as he does repeatedly.⁷ What analysis of Bancroft's work does regarding a corroboration of MacCallum's work has already been shown.

SUMMARY.

Moderate doses of the saline cathartics exert no purgative action when injected subcutaneously or intravenously.

Large doses similarly administered are dangerous and often fatal.

When given in large quantities intravenously, the fluid feces resulting cannot be attributed offhand to a purgative action of

¹ Bancroft: *loc. cit.*, p. 198.

² MacCallum: *loc. cit.*, p. 102-103, p. 106.

³ Bancroft: *loc. cit.*, p. 199, pp. 201-204.

⁴ MacCallum: *loc. cit.*, p. 103.

⁵ Bancroft: *loc. cit.*, p. 196.

⁶ Bancroft: *loc. cit.*, pp. 191-192.

⁷ *Ibid.*, p. 193, p. 206, p. 210, p. 211.

the salt injected, the increased fluidity of the gut contents being due partly or entirely to an increased secretion and transudation of the glands of the gut in their attempt to aid the overwhelmed kidneys. Physiological saline solution infused in large quantities has the same action (Cohnheim and Lichtheim).

Normal rabbits show such marked variations in the total hourly output of feces that at least four controls should be used in testing for the minimal purgative effect of any substance.

Bancroft's statement that controls in my previous experiments would have demonstrated a purgation in the experimental animals is unintelligible, for a majority of those rabbits passed no feces for one hour after injection of the salines;¹ controls could not do less.

Bancroft's own work does not justify his own conclusions, nor does it corroborate MacCallum's results:

(a) The calculation that the subcutaneous injection of small doses of sodium citrate increases the fecal output to twenty-three times that of normal animals is wrong, for it is based on the output of artificially constipated controls.

The passage of a few grams of dry feces, in amount well within the limits of normal variations, should not be called a purgation (13 out of 16 of his experiments showed less than 8 grams of feces in 3 hours); such a "purgation" has neither scientific nor practical interest.

(b) His second series with subcutaneous injections of small doses demonstrates no purgation whatsoever.

(d) The subcutaneous or intravenous injection of toxic and lethal doses of the salines is irrelevant to the question at issue, as obviously the injections must be as harmless as when administered by mouth.

Note added at proof correction.

While this article was in press, a paper by Frankl, from Hans Meyer's laboratory, appeared in the *Archiv für experimentelle Pathologie und Pharmacologie* (lvii, Heft 5 u. 6, p. 386) which deals with the effect of sodium sulphate when injected intravenously. Frankl observed no purgation, but rather a moderate constipation, a result perfectly in accord with mine.

¹ According to MacCallum (*loc. cit.*, p. 103) the purgation usually occurs during the first hour.

CHANGES IN THE BILE OCCURRING IN SOME INFECTIOUS DISEASES.

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The causes of gallstone formation were but little understood until about twenty years ago, when it was recognized by Budd,¹ by Bristowe² and by Naunyn³ that the most of the cholesterin in the bile was derived not from the blood, but from the degeneration of the epithelium lining the gall-bladder and the biliary passages and that the formation of cholesterin gallstones is due to a local affection of these mucous membranes rather than to any constitutional disturbance.

In the early study of the causes of gallstone formation, it was noted that calculi were frequently deposited as a result of "fevers." Gilbert and Girode⁴ in 1890 found that the biliary passages can be invaded by the bacillus of Escherich and that this bacillus is one of the causes of angio-colitis, and of suppurative cholecystitis. Following this discovery, cultures of *B. typhosus*, *B. coli communis*, of staphylococci, of streptococci and other bacteria were made from the bile by many observers.⁵

¹ Budd: *Med. Press and Circular*, Nov. 16, 1892.

² Bristowe: *The Lancet*, Feb. 19, 1887.

³ Naunyn: *Klinik der Cholelithiasis*, Leipsig, 1892.

⁴ Gilbert and Girode: *Compt. rend. de la soc. de biol.*, 27 déc., 1890.

⁵ For references on the bacteria found in the bile, see, Lippmann: *Le microbisme biliaire normal et pathologique*, Paris, 1904; Clairmont: *Wiener klin. Wochenschr.*, xii, p. 1068, 1899; Kelly: "Infections of the Biliary Tract," *Amer. Journ. of Med. Sciences*, cxxxii, p. 446, 1906; Lartigau: "The Relation of Bacteria to the Development of Gallstones," *Calif. State Journ. of Med.*, iv, pp. 17-21, 1906; Letienne: "Recherches bacteriologiques sur la bile humaine," *Arch. de méd. exp.*, iii, p. 761, 1891.

Mignot,¹ Gilbert and Fournier,² Cushing³ and Richardson⁴ succeeded in causing the formation of stones, by introducing into the gallbladders of animals cultures of the abovenamed organisms. A majority of the studies which have been made of gallstone formation have followed Gilbert and Fournier's work in trying to determine the relation of bacterial invasion to the formation of calculi.

During the winters of 1903 and 1904, I had the opportunity of examining a number of specimens of bile (225 in all)⁵ which were obtained from autopsies from the City, Bellevue, Presbyterian and Hood Wright hospitals, through the kindness of the pathologists in these laboratories. This work was done under a grant from the Rockefeller Institute. By estimating the cholesterin and calcium present and by studying the physical characteristics of the different specimens, an attempt was made to determine the relations between certain pathological conditions in the bile itself and the deposition of stones.

The normal bile is dark green, or dark brown or reddish brown in color and has a neutral or slightly alkaline reaction. It has a viscid consistence, the viscosity being due to the presence of a nucleo-proteid and not to mucus. The average amount of solids present, as determined in this series of specimens of bile was 11.6 per cent. There is no free cholesterin present in the normal bile. There is but little sediment, and this is made up of particles of granular epithelial débris and of the characteristic columnar epithelial cells lining the gallbladder. The cells are finely granular and nucleated, with their margins well defined. Small patches of epithelial cells are frequently detached from the wall of the gallbladder postmortem and are found floating in the bile.

In the routine examinations of specimens of bile, it is noteworthy that so large a number are definitely abnormal. Of the 225 specimens examined in this series, 72 (or nearly one in three)

¹ Gilbert and Fournier: *Bull. soc. biol.*, Oct. 30, 1897.

² Mignot: *Recherches expérimentales et anatomiques sur les cholecystitis*, Thèse, Paris, 1896.

³ Cushing: *Johns Hopkins Hospital Bulletin*, x, p. 166, 1899.

⁴ Richardson: "On the Rôle of Bacteria in the Formation of Gallstones," *Journ. of the Boston Society of Medical Sciences*, iii, p. 79, 1899.

⁵ While these cases did not form a successive series, they were not selected cases.

showed definite morbid changes in the bile, although the symptoms were so slightly marked that a diagnosis of cholecystitis or of gallstone formation had been made in only two cases. In seventeen of the specimens there were evidences of an early stage of cholecystitis, fourteen contained bilirubin calcium calculi, and forty-four contained free cholesterin either as (1) amorphous granular masses mixed with epithelium, (2) free cholesterin crystals, (3) cholesterin calculi or mixed gallstones, or (4) deposits of cholesterin crystals in the mucous membrane of the gallbladder. Leucocytes were found in seven specimens and blood in two.

That the typhoid bacillus commonly passes into the gallbladder in typhoid fever and that gallstones may be the result of such an infection has been definitely proven. Flexner found the typhoid bacillus in the bile in 58 per cent of fatal cases of typhoid fever. Halstead obtained a history of typhoid fever in one-third of the cases operated on for gallstones. Gilbert and Fournier by injecting attenuated cultures of typhoid bacilli into the gallbladders of dogs succeeded in producing calculi.

The infection of typhoid fever differs from that of most specific fevers in that the specific organism is present in large numbers in the intestine, and so can gain entrance into the gallbladder not only through the general circulation but also through the common duct and through the portal vein, or, if the peritoneum is infected, it may enter through the wall of the gallbladder. Colon bacilli and anaërobic forms of intestinal bacteria are also commonly found in the bile. Lartigau has found by experiments upon animals that pathogenic bacteria introduced into the intestines find their way into the gallbladder. The bacterial examination of the bile at autopsies and during operations has shown that in other infectious fevers, in which the intestine is not the seat of the bacterial invasion, microorganisms may pass into the gallbladder through the bile. There has, however, been but little emphasis laid on the frequency of gallbladder involvement in infectious fevers or on the danger of gallstone formation as a consequence of this complication.

In the series of cases noted above, fifty-one of the patients died of some infectious disease and in seventeen there were characteristic changes in the bile which were not noted in patients dying from chronic diseases.

The bile in these cases presents to the naked eye a purulent appearance. It is usually red or reddish brown or a muddy brown color, having a lighter shade than normal from the large number of cells suspended in it. It is generally acid in reaction although it may be alkaline. The percentage of solids present is practically normal, the average of all the cases being 10.3 per cent. The percentage of cholesterin is a little above the normal. The bile as a rule has lost all viscidty, having a creamy consistency. A point with reference to these specimens which is especially noteworthy, is that while to the naked eye they appear to contain pus, on microscopical examination the cells present are found to be not leucocytes but solitary columnar cells from the mucous membrane of the gallbladder. In any drop the field is found crowded with these single epithelial cells. There are also some cells in patches. Cases showing this characteristic appearance in the bile were two of typhoid fever, ten of pneumonia and one each of fibrino-purulent peritonitis, puerperal septicæmia, acute parenchymatous nephritis, chronic endocarditis with fever and abscess of the liver.

Another distinctive type of cystic bile, which would seem to be a later stage of that just described, was found in twelve of the specimens. This type was also found only in acute infectious diseases, excepting one case of lead poisoning. The bile has in these specimens the appearance of a soft dark mud. It is thick in consistency and filled with brown particles. On examination with the microscope the sediment is found to contain degenerated epithelial cells and amorphous granular masses, stained yellow, which give a marked reaction for cholesterin. The total solids in these specimens are increased, the average being 13.1 per cent. The cholesterin is markedly increased. The reaction varies, being in some instances alkaline in others acid. The cases in which the bile showed these characteristics were five of pneumonia, and one each of empyema following pneumonia, typhoid fever, general purulent peritonitis complicating gastric ulcer, purulent meningitis secondary to mastoid abscess, degenerating fibroid of the uterus, general miliary tuberculosis and lead poisoning.

Solitary cholesterin crystals were found in the bile in fourteen of the specimens. These were usually associated either with the soft yellow granular masses which are made up of degenerating

epithelium, or with calculi or with both. In some cases of chronic endocarditis or endarteritis, a small number of cholesterol crystals were found.

A degeneration of the epithelial cells lining the gallbladder, characterized by a deposit of cholesterol in fine points or granules, may occur while the cells are still attached to the mucous membrane. Brockbank¹ speaks of having in two cases seen these small specks made up of cholesterol crystals lying apparently in retention cysts, in the mucous membrane. These deposits in the wall of the gallbladder seem not to be uncommon and may escape notice because of the ready solubility of the cholesterol in the ether used in dehydrating specimens. The mucous membrane of the gallbladder presents numerous depressions or alveoli having a fine honeycomb appearance, and the crystalline deposits may form in lines following the walls of these depressions. They are usually not removed by gentle scraping with a spatula. In one hundred and fifty-four of the above cases the wall of the gallbladder was examined for these deposits and they were found in sixteen cases or 10 per cent. Nine of these cases were of acute diseases—lobar pneumonia or septic infections—one was of Basedow's disease in a woman twenty-eight years of age, the remaining six were cases of nephritis or endarteritis and five of the patients were over fifty years of age. In five specimens with deposits in the wall of the gallbladder there were solitary cholesterol crystals in the bile, five others contained granular degenerating masses of epithelium and one contained cholesterol stones apparently recently formed.

Bile pigment alone or associated with but little cholesterol was found in fifteen of the specimens examined, that is, in 1.2 per cent. The bile pigment, which is made up chiefly of bilirubin calcium, was precipitated in numerous microscopic needle-shaped spicules or in small calculi. This deposit of bilirubin calcium alone was not noted in any acute cases and with one exception it occurred in patients over fifty years of age. The reaction of the bile was found to be neutral or acid in the cases with bilirubin calcium deposits in which it was tested. This suggests that a change to an acid reaction leads to a precipitation of bilirubin calcium

¹ Brockbank: *On Gallstones*, p. 72, 1896.

from the bile. Kramer,¹ working at the Rockefeller Institute, found that after introducing cultures of typhoid bacilli or of colon bacilli into portions of filtered bile, the reaction became acid and pigment with a few cholesterin crystals was precipitated. He concluded that the acid reaction was the cause of the precipitation and that the formation of gallstones, after the gallbladder was infected with typhoid or colon bacilli, might be due to the change in reaction rather than to an excessive cholesterin formation. This is doubtless true in some cases, when the pigment deposit occurs alone or with a slow deposit of cholesterin around it, as is found in certain chronic diseases.

A histological examination of the wall of the gallbladder was made by Dr. Mercelis, in twenty-nine of the cases having acute infectious diseases. In twelve of these there was found a recent inflammation. In fifteen cases a chronic inflammation already existed. The histological changes in these cases were those of an early stage of a subacute or chronic inflammation. The mucosa and sometimes the deeper tissues showed an increased infiltration with lymphocytes. This was usually most marked at the free border of the mucosa. Pus cells, if present at all, were in very small numbers. Congestion with few exceptions was absent or moderate. The same was true of œdema. The condition of the epithelium varied, sometimes it was found in good condition, sometimes degenerated, and sometimes the surface was denuded.

The result of this study of the bile under varying pathological conditions is suggestive rather than definite. The original purpose of the investigation was to determine the percentage of cholesterin in the bile, in different diseases and the other observations were incidental to this. A quantitative determination of the cholesterin was made on the most of the specimens examined. But it was found that the method used, that of Ritter, which had seemed to be an accurate one, was unreliable as it had been carried out in these experiments, and that only general conclusions could be drawn from the observations. With regard to the cholesterin content of the bile the following facts were noted:

¹ Kramer: "The Pathogenesis of Gallstones." *Journ. of Exp. Med.*, ix, p. 319, 1907.

That in an early stage of cholecystitis, where there is free drainage from the gallbladder, and the epithelial cells are but slightly disintegrated, there is a slight increase in the cholesterin content of the bile.

That where there is an obstruction to the flow of bile from the gallbladder and the bile is filled with masses of degenerating epithelium, there is a marked increase in the cholesterin.

That where there is an excess of cholesterin in the contents of the gallbladder, the most of this increase over the normal amount is held in suspension rather than in solution.

Apart from these observations on the cholesterin in the bile, the most significant fact noted was that cholecystitis in a mild or more marked degree is a common complication, not only of typhoid fever, as has been frequently observed, but also of pneumonia and of suppuration in other parts of the body. Usually this is so slight that it gives no distinctive symptoms but a histological study of the wall of the gallbladder shows the beginning of a chronic inflammation and on examination of the bile, there is found a marked desquamation of the epithelial cells of the mucous membrane. If the cystic duct is freely patulous, these cells will be discharged into the intestine, but if there is a tendency to obstruction or if the desquamation of the epithelial cells is excessive, there will be a retention of degenerating epithelial elements in the gallbladder with the formation of cholesterin, or eventually the production of gallstones.

A SYNTHESIS OF CERTAIN NATURALLY OCCURRING ALIPHATIC KETONES, WITH A SUGGESTION OF A POSSIBLE MODE OF FORMATION OF THESE SUBSTANCES IN THE ORGANISM.

(METHYL-*n*-NONYL KETONE, METHYL-*n*-HEPTYL KETONE, METHYL-*n*-AMYL KETONE.)

By H. D. DAKIN.

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In a previous paper¹ it was shown that butyric acid when oxidized in the form of its ammonium salt with hydrogen peroxide yields among other products, aceto-acetic acid, which undergoes further decomposition with formation of acetone and carbon dioxide:



This reaction had special interest since it furnished an example of a type of β -oxidation which is commonly met with in the breakdown of fatty acids in the organism, but which has seldom been encountered in reactions occurring outside the body.

Although, so far as is known, acetone is the only simple aliphatic ketone of importance in connection with animal metabolism, several members of this class of substances are met with as important constituents of certain essential oils. Thus, methyl-*n*-nonyl ketone, $\text{CH}_3(\text{CH}_2)_8\text{CO.CH}_3$, is the principal constituent of oil of rue from *Ruta graveolens*,² and is also found in the essential oil of lime leaves from *Citrus limetta*.³ Methyl-*n*-heptyl ketone, $\text{CH}_3(\text{CH}_2)_6\text{CO.CH}_3$, is the main constituent of Algerian oil of rue⁴ and it also occurs in smaller quantity in ordinary oil of rue

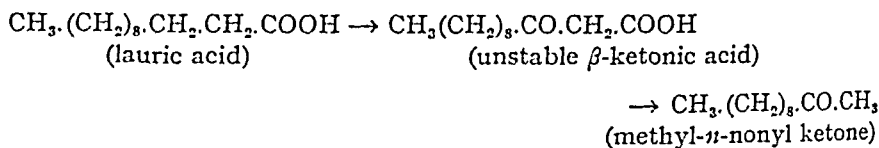
¹ This *Journal*, iv, p. 77, 1908.

² Greville Williams: *Phil. Trans.*, i, p. 99, 1858; Houben: *Ber. d. deutsch. chem. Gesellsch.*, xxxv, p. 3587; Thoms: *Ber. d. deutsch. pharm. Gesellsch.*, xi, p. 3.

³ Watts: *Trans. Chem. Soc.*, xlix, p. 316.

⁴ v. Soden and Henle: *Pharm. Zeit.*, xlvi, pp. 227 and 1026.

from other sources,¹ and has been found in the essential oil of cloves.² Methyl-*n*-amyl ketone, $\text{CH}_3(\text{CH}_2)_4\text{CO.CH}_3$, has been detected in oil of cloves³ and in cinnamon oil from Ceylon.⁴ It was obviously of interest to see whether these ketones might be prepared by oxidation of the corresponding saturated fatty acid, in a manner exactly analogous to the formation of acetone by the oxidation of butyric acid. Experiment showed that this could be easily accomplished. Thus lauric acid on conversion into its ammonium salt and warming with hydrogen peroxide readily yields methyl-*n*-nonyl ketone. There can be but little doubt that an unstable β -ketonic acid is the precursor of the ketone and that this loses carbon dioxide in the usual way:



In an entirely similar fashion methyl-*n*-heptyl ketone was obtained by the oxidation of capric acid and methyl-*n*-amyl ketone by the oxidation of caprylic acid. The ketones in each case were converted into crystalline derivatives and found to be identical with the natural products. Experiments indicate that this reaction is a perfectly general one for preparing methyl ketones from saturated fatty acids and will be further investigated.

The methods hitherto employed for the synthesis of the naturally occurring aliphatic ketones⁵ afford no basis for speculation as to possible modes of formation in the organism and so far I have been unable to find any reference to this particular point. Although at present direct information is wanting, I think that there are a number of reasons which make it not improbable that the formation of ketones by oxidation of saturated fatty acids with hydrogen peroxide is a reaction very similar to that which

¹ Thoms and Houben: *loc. cit.*

² Schimmel's *Geschäftsberichte*, April, 1903.

³ *Ibid.*, April, 1897, and 1902.

⁴ *Ibid.*, April, 1902; Walbaum and Hühlig: *Journ. f. prakt. Chem.*, [2], lxvi, p. 47.

⁵ See R. Meldola: *The Chemical Synthesis of Vital Products*, p. 200.

may occur in the plant organism. The evidence upon which the suggestion is based may be arranged as follows:

(i) The reaction is analogous to the formation of acetone from butyric acid—a change known to occur under certain conditions in the animal body and which can be artificially imitated with hydrogen peroxide.

(ii) The principal naturally occurring aliphatic ketones are *methyl* ketones, a fact that harmonizes with the view that they may be formed from β -ketonic acids by loss of carbon dioxide.¹

(iii) The naturally occurring ketones have an *uneven* number of carbon atoms, corresponding to their formation from saturated fatty acids with *even* numbers of carbon atoms. Higher fatty acids with uneven numbers of carbon atoms are found very rarely in nature.

(iv) Saturated fatty acids, with even numbers of carbon atoms are actually found in the natural oils which contain aliphatic ketones.

(v) An ever increasing number of reactions emphasize the close similarity between oxidations carried out with hydrogen peroxide under certain conditions and those occurring in living tissues.

It would be of interest to test the above hypothesis experimentally by trying to trace a relationship between the quantity of fatty acids and of ketones present at various periods in the course of development of some of the plants yielding essential oils in which ketones predominate.

EXPERIMENTAL.

In each case the pure fatty acid (1 mol.) was neutralized with a slight excess of ammonia and slowly distilled with 3 per cent hydrogen peroxide (2 mols.). The solutions must be contained in large flasks as much foaming occurs. The first portions of the distillate contain a quantity of suspended oil equal to about 5–8 per cent of the amount of acid taken for oxidation. The distillate is stopped as soon as no more oil comes over and a further quantity of peroxide with a little ammonia is then added,

¹ α and γ -ketonic acids do not undergo a similar change.

and on redistillation an additional amount of ketone is obtained. This process may be repeated many times but the yield of ketone becomes progressively less. The oily ketone in each case was separated from the aqueous portion and washed with water to remove ammonia. The aqueous portion contains lower fatty acids and a substance giving the reactions of acetaldehyde. These products will be more carefully examined. The crude ketones were redistilled and identified by converting them into their semicarbazones.

Methyl-*n*-nonyl ketone, prepared from lauric acid, on treatment with semicarbazide acetate in the usual way gave a crystalline semicarbazone, which after crystallization from alcohol was obtained in the form of fine shining platelets melting at 122° – $122\frac{1}{2}^{\circ}$. The properties of the compound agreed in every way with that described by Power and Lees.¹ In addition the paranitrophenylhydrazone was prepared by treating an alcoholic solution of the ketone with paranitrophenylhydrazine dissolved in excess of 40 per cent acetic acid. The product crystallized from alcohol in yellow needles, frequently aggregated into rosettes, melting sharply at 90° – 91° . On analysis:

0.1722 gram gave 0.4226 gm. CO ₂ and 0.1368 gm. H ₂ O.		
	Calculated for C ₁₇ H ₂₇ N ₃ O ₂ :	Found:
H	8.85 per cent.	8.83 per cent.
C	66.88 " "	66.82 " "

For comparison the same compound was prepared from methyl-*n*-nonyl ketone, from natural sources. It melted at 90° – 91° and was identical in every way with the compound previously obtained. On analysis:

0.1122 gm. gave 0.2754 gm. CO ₂ and 0.0915 gm. H ₂ O.		
	Calculated for C ₁₇ H ₂₇ N ₃ O ₂ :	Found:
H	8.85 per cent.	9.06 per cent.
C	66.88 " "	66.94 " "

The methyl-*n*-heptyl ketone, obtained by the oxidation of capric acid, was identified by conversion into its semicarbazone, which after crystallization from alcohol melted sharply at 118° –

¹ *Trans. Chem. Soc.*, lxxxi, p. 1588.

119°, agreeing with the product obtained by Thoms and by Soden and Henle, who prepared the semicarbazone from naturally occurring ketone. The methyl-*n*-amyl ketone, derived from the oxidation of caprylic acid gave a semicarbazone melting at 122°–123° identical with the product obtained from methyl-*n*-amyl ketone from Ceylon cinnamon oil.

A COMPARATIVE STUDY OF THE OXIDATION OF THE AMMONIUM SALTS OF SATURATED FATTY ACIDS WITH HYDROGEN PEROXIDE.

(PRELIMINARY COMMUNICATION.)

By H. D. DAKIN.

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(Received for publication, January 9, 1908.)

The statement that a knowledge of the mechanism of the reactions involved in the tissue oxidation of fats or fatty acids would be of the utmost importance for the progress of physiological chemistry cannot be gainsaid; but the difficulties attending efforts to obtain an insight into these processes are so great that up to the present but little progress has been made. The most promising method of attacking the problems would doubtless be on the lines of physiological experiments based upon an adequate knowledge of the reactions by which the fatty acids may be made to liberate their energy outside the body, under conditions not too far removed from those prevailing in the living cell. Unfortunately, our information upon the breakdown of the fatty acids outside the body is of a fragmentary character and needs to be much enlarged before it will be possible to draw many deductions concerning the possible course of fatty acid catabolism in the living cell. Emil Fischer, in his recent Faraday lecture,¹ writes: "Still less is known of the manner in which fats undergo combustion to carbon dioxide and water in the animal body. Hitherto this change has only been effected at high temperatures. It is obvious that it would be of far greater interest to discover oxidizing agents which would produce the effect at low temperatures. No doubt many intermediate products would be met with and the study of these would afford valuable assistance to biologists in devising experiments with animals."

¹ *Trans. Chem. Soc.*, xci, p. 1754, 1907.

On examining the existing literature upon the oxidation of the saturated fatty acids it is found that the reagents most commonly employed have been potassium permanganate, chromic acid and concentrated nitric acid. The results obtained by the use of these powerful reagents, although of chemical interest, are difficult to correlate with biological changes for the former reactions are frequently violent and are accompanied by abrupt liberation of energy and moreover the products are frequently such as resist further oxidation.¹

It was clearly of interest to study more closely the oxidation of the fatty acids employing more gentle oxidizing agents than those that have been employed hitherto. In a number of recent communications it has been shown that many oxidations effected under certain conditions with hydrogen peroxide bear an extremely close similarity to certain biochemical oxidations, especially as regards the oxidation of amino-acids, aromatic acids, butyric acid and other substances playing important biochemical rôles.² It was therefore determined to investigate the action of hydrogen peroxide upon the fatty acids, or rather their soluble ammonium salts, and as a result the surprising fact was discovered that even the stable higher fatty acids, such as palmitic and stearic acids were attacked to a very considerable extent by this reagent. In the case of the lower fatty acids the reaction progresses to some extent at ordinary temperatures and it is possible that this is also true of the higher fatty acids. At any rate it is probable that with a suitable catalyst, the reaction would proceed in all cases at low temperatures.

¹In general it may be said that the oxidation of the higher fatty acids with the usual chemical reagents results principally in the production of di-basic acids together with smaller quantities of monobasic acids. For example, myristic acid ($C_{14}H_{28}O_2$) on long continued boiling with nitric acid (sp. gr. 1.3) yields varying proportions of suberic, pimelic, adipic, glutaric, succinic and oxalic acids. Palmitic acid on oxidation with alkaline permanganate yields adipic, succinic and oxalic acids together with caproic, butyric and acetic acids. There is at present no reason to believe that the higher dibasic acids are intermediary products in the catabolism of the higher fatty acids and in the absence of such information, their formation as oxidation products of the higher fatty acids has limited biological significance.

²Dakin: *This Journal*, i, pp. 171 and 271; iii, p. 419; iv, pp. 63, 77 and 91.

In some cases considerable progress has been made in determining the course of the reaction but the complete study of the products from such a large number of fatty acids is a task of some magnitude and time will be necessary before all the details can be obtained. In the meantime estimations have been made of the product that is common to all the fatty acids, namely, carbon dioxide. The results are collected in the form of a table and record the amount of carbon dioxide in grams obtained by the oxidation of $\frac{1}{100}$ g. mol. of each of the fatty acids with $\frac{2}{100}$ g. mol. of 3 per cent hydrogen peroxide under certain arbitrary conditions described below. The yield of carbon dioxide is of course not nearly the maximum amount obtainable as will be seen later.

TABLE I

Estimations of Carbon Dioxide formed by Oxidation of $\frac{1}{100}$ g. mol. of the Ammonium Salts of Saturated Fatty Acids with $\frac{2}{100}$ g. mol. 3 per cent H_2O_2 .

Acid.	Weight of acid.	Yield of carbon dioxide	Apparent percentage oxidation of acid.	Products other than carbon dioxide and water.
	grams.	gram.		
Formic.	0.46	0.209	47.5	
Acetic.	0.60	0.089	20.2	Glyoxylic acid, formaldehyde, formic acid and its oxidation products.
Propionic.	0.74	0.191	43.4	Acetaldehyde, acetic acid and its oxidation products.
n-Butyric.	0.88	0.094	21.4	Aceto-acetic acid, acetone, propionic aldehyde, propionic acid and its oxidation products.*
Isobutyric.	0.88	0.104	23.6	Acetone.
n-Valeric.	1.02	0.073	16.4	
Isovaleric.	1.02	0.068	15.5	Acetone.
n-Caproic.	1.16	0.042	9.5	
n-Heptylic (Oenanthic)	1.30	0.041	9.3	Incompletely investigated—but in most cases evidence obtained of formation of lower fatty acids and of ketones, derived from β -ketonic acids, $R.CO.CH_2COOH$, by loss of carbon dioxide. (See following paper.)
Caprylic.	1.44	0.055	12.5	
Nonylic (Pelargonic) ...	1.58	0.040	9.1	
Capric.	1.72	0.054	12.3	
Lauric.	2.00	0.034	7.8	
Myristic.	2.28	0.044	10.0	
Palmitic.	2.56	0.043	9.8	
Stearic.	2.84	0.039	8.9	

*This volume, p. 77.

The estimations of the yield of carbon dioxide were made by taking $1\frac{1}{10}$ g. mol. of the fatty acid and adding ammonia until the reaction was decidedly alkaline.¹ The solution was boiled for a few moments to remove carbon dioxide and cooled, and approximately 3 per cent hydrogen peroxide of known strength was then added in amount equal to $1\frac{2}{10}$ g. mol. The mixture was contained in a flask connected with a small wash-bottle containing sulphuric acid to remove ammonia and then with absorption bulbs containing standard baryta solution. A current of washed carbon-dioxide-free air was aspirated through the apparatus throughout the course of the experiment. The contents of the flask were gently heated by means of a water-bath which was eventually heated to boiling. Each experiment was continued for at least two hours but in some cases, notably with the higher fatty acids in which much foaming was encountered, the experiment had to be somewhat prolonged. At the close of the experiment the contents of the absorption bulbs were titrated with $\frac{N}{4}$ hydrochloric acid using phenolphthalein for indicator.

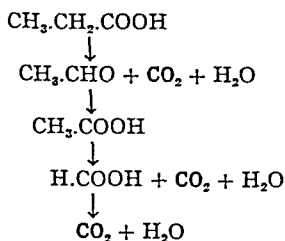
In most cases carbon dioxide was evolved rapidly a few minutes after the commencement of the experiment and most of the carbon dioxide was liberated at the end of half an hour. It should be clearly understood that no claim to great accuracy is made and that the results are intended to be merely comparative and do not represent the maximum amount of carbon dioxide obtainable under other conditions. By increasing the proportion of hydrogen peroxide added, the yield of carbon dioxide is increased but even when very large amounts of peroxide are added the oxidation is never complete. The following figures illustrate the influence of varying proportions of peroxide upon the yield of carbon dioxide obtainable from acetic acid:

Acid taken.	3 per cent hydrogen peroxide.	Yield of carbon dioxide.
	<i>g. mol.</i>	<i>gram.</i>
$1\frac{1}{10}$ g. mol = 0.60 gram	$1\frac{1}{10}$	0.040
"	$1\frac{2}{10}$	0.089
"	$1\frac{3}{10}$	0.129
"	$1\frac{4}{10}$	0.172
"	$1\frac{6}{10}$	0.180

¹ In the case of the higher fatty acids, whose ammonium salts readily undergo hydrolytic dissociation, ammonia was added in amount equal to an excess of about 25 per cent over the quantity theoretically necessary.

Most of the figures representing the yield of carbon dioxide in Table I, represent the mean of several experiments agreeing fairly closely. It should be mentioned that a large excess of ammonia affects the oxidation prejudicially and also that it is always necessary to perform a blank experiment with each batch of hydrogen peroxide, since frequently the reagent contains large amounts of carbon dioxide. Hydrogen peroxide containing small amounts of carbon dioxide may be safely employed if a suitable correction be applied.

Consideration of the results shown in Table I. If the reaction involving the oxidation of the ammonium salt of the fatty acid were limited to the evolution of one molecule of carbon dioxide from each molecule of fatty acid, the theoretical yield of carbon dioxide in each experiment would be 0.44 gram. As a matter of fact however the reaction in many cases proceeds further, as is seen, for example, in the oxidation of propionic acid which, neglecting some of the intermediate products, breaks up according to the following scheme:



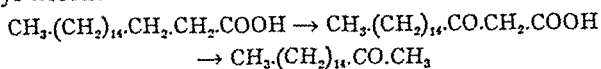
Even when no excess of hydrogen peroxide be employed it is possible to demonstrate that all of these reactions have progressed to some extent, so that it is impossible to calculate exactly, the proportion of fatty acid which has undergone oxidation on the basis of the yield of carbon dioxide. The error in such a calculation is certainly much less for the higher members of the series than for the lower and as the figures have a considerable interest it has been deemed justifiable to calculate what may be termed the apparent percentage oxidation of the fatty acids investigated. It will be seen that under the special conditions of the experiment that formic acid and propionic acid are each decomposed to the extent of between almost 50 per cent. Acetic acid

on the other hand seems to be less readily oxidized and yields results approximately the same as those given by the normal and *iso*-butyric acids. Normal and isovaleric acid appear to be slightly less readily oxidized (15–16 per cent) while the remaining acids up to and including stearic acid show no very marked variations among themselves and all undergo oxidation to the extent of about 8–12 per cent. In general, it may be said that the acids up to and including caproic acid, with the exception of propionic acid exhibit a tendency to be less easily oxidized with rise in molecular weight. The acids from heptylic to stearic show comparatively small variations among themselves. It must be borne in mind that the numbers representing the percentage decomposition of the various acids are only approximate and of merely comparative value.

The identification of the products of oxidation, other than carbon dioxide and water is a task of considerable magnitude and much remains to be done in this direction. *Formic acid* can obviously yield only carbon dioxide and water. The oxidation of *acetic acid* with hydrogen peroxide under slightly different conditions from those prevailing in my own experiments has been made by Hopkins and Cole, who identified glyoxylic acid, formaldehyde and formic acid. *Propionic acid* (1 g. mol.) when neutralized with ammonia and slowly distilled with hydrogen peroxide (2 g. mol.) yields much acetaldehyde, acetic acid and a little formic acid. The acetaldehyde was identified as the p-nitrophenylhydrazone (m. p. 128° – $128\frac{1}{2}^{\circ}$) and by the usual qualitative reactions, while the presence of acetic acid was determined by analysis of the silver salt after removal of formic acid. The products of oxidation of *butyric acid* have already been investigated and the results presented in a separate communication.¹ *Iso*-butyric acid on oxidation yields acetone in large quantity, traces of formic acid, but no aldehydes. The acetone was identified by converting it into the paranitrophenylhydrazone which after recrystallization from alcohol melted at 149° . *Iso*valeric acid yields acetone but the products of oxidation of normal valeric acid have not yet been investigated. The higher fatty acids from caproic to stearic on oxidation yield at least in part ketones

¹ This *Journal*, iv, p. 77, 1908.

derived from β -ketonic acids by loss of carbon dioxide. Substances giving the reactions of acetaldehyde and lower fatty acids are formed simultaneously. Thus stearic acid yields quindekyl-methyl-ketone

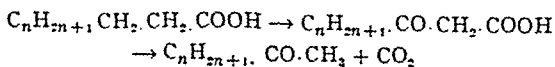


This reaction appears to be a general one and will be further investigated especially as it appears to have considerable biochemical significance (cf. the following paper). The reaction appears to be completely analogous to the formation of aceto-acetic acid and acetone from butyric acid under similar conditions.

SUMMARY.

The ammonium salts of the saturated fatty acids from formic to stearic acid are comparatively easily oxidized by means of hydrogen peroxide. In some cases the reaction progresses to some extent at room temperatures. In all cases carbon dioxide is liberated. Table I contains the results of estimations of the carbon dioxide evolved when $\frac{1}{100}$ g. mol. of the fatty acid is treated under certain arbitrary conditions with $\frac{2}{100}$ g. mol. of approximately 3 per cent hydrogen peroxide. The results appear to indicate that formic acid is most readily oxidized, followed by propionic acid. Next in order come acetic acid, butyric and isobutyric acids which yield approximately the same results and are somewhat more readily oxidized than valeric or isovaleric acid. The remaining acids show but slight variations among themselves and yield approximately $\frac{1}{100}$ g. mol. of carbon dioxide for every gram-mol. of fatty acid taken for oxidation.

Propionic acid on oxidation under the prescribed conditions yields acetaldehyde, acetic acid, and a little formic acid. Isobutyric acid and isovaleric acid both yield acetone. The higher fatty acids from caproic to stearic are oxidized, at least in part, with formation of ketones, derived from β -ketonic acids, according to the following scheme:



Lower fatty acids and aldehydes are formed simultaneously. The reactions appear to have some biochemical relationships and will be further investigated.

NOTE ON THE USE OF PARANITROPHENYLHYDRAZINE FOR THE IDENTIFICATION OF SOME ALIPHATIC ALDEHYDES AND KETONES.

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In the course of studies upon the products of oxidation of the amino-acids, saturated fatty acids and various oxy-acids with hydrogen peroxide it was necessary to employ methods for the identification of small quantities of the simple aldehydes and ketones. At first considerable difficulty was experienced in obtaining satisfactory crystalline derivatives for in most cases phenylhydrazine and many of its derivatives give oily hydrazones or yield crystalline products with difficulty. By employing paranitrophenylhydrazine these difficulties have disappeared as this substance reacts promptly and practically quantitatively with all the simple aldehydes and ketones giving derivatives which are practically insoluble in water and which are characterized by the great ease with which they may be crystallized and obtained in a state of purity.

Paranitrophenylhydrazine is readily obtainable and has the desirable quality of keeping almost indefinitely. It is usually prepared by the reduction of the product of the diazotization of paranitraniline and crystallizes in glistening orange-red platelets melting at 157° . Paranitrophenylhydrazine has already been made use of by a number of observers for the characterization of aldehydes and ketones but most of the simple derivatives have not yet been described.

The following derivatives were all prepared by dissolving the aldehyde or ketone in a little water or alcohol and adding to the clear solution a slight excess of a cold filtered solution of paranitrophenylhydrazine in about 30 parts of 40 per cent acetic acid. Precipitation of the hydrazone is immediate and the product almost invariably crystallizes at once. The precipitate is

filtered off, washed with a little dilute alcohol and then recrystallized. Alcohol is the most generally useful solvent, although occasionally a mixture of benzene and petroleum is of service. The following table contains the melting point of nitrophenylhydrazones prepared in this way. For the sake of completeness the derivatives of formaldehyde, acetaldehyde and acetone, which have already been prepared by Bamberger and Hyde are included.¹ All the hydrazones referred to below dissolve in alkali with an intense dark red color.

Formaldehyde paranitrophenylhydrazone	melts at	181°-182°
Acetaldehyde	"	" 128°-128½°
Propionic aldehyde	"	" 123°-124°
<i>n</i> -Butyric aldehyde	"	" 91°- 92°
Isobutyric aldehyde	"	" 131½°-132°
Isovaleric aldehyde	"	" 109°-110°
Glyoxylic acid	"	" c200°
Acetone	"	" 149°
Methylethyl ketone	"	" 128°-129°
Methylisopropyl ketone	"	" 108°-109°
Methyl- <i>n</i> -nonyl ketone	"	" 90°-91°

Propionic aldehyde. The paranitrophenylhydrazone was prepared from propionic aldehyde obtained by the oxidation of propylalcohol with chromic acid and also from aldehyde from the oxidation of α -oxy-isobutyric acid with hydrogen peroxide.² The compound is readily soluble in cold alcohol and crystallizes in needles. On analysis:

0.1110 gm. gave 0.2290 gm. CO₂ and 0.0565 gm. H₂O.

	Calculated for C ₉ H ₁₁ N ₃ O ₂ :	Found:
C.....	56.0 per cent.	56.2 per cent.
H.....	5.7 " "	5.7 " "

n-Butyric aldehyde was prepared by the distillation of calcium butyrate and calcium formate according to Lipp's directions,³ and also by the oxidation of α -amino-*n*-valeric acid with hydrogen

¹ Bamberger and Sternitzki: *Ber. d. deutsch. chem. Gesellsch.*, xxvi, p. 1306; Bamberger: *ibid.*, xxxii, p. 1807; Hyde: *ibid.*, xxxii, p. 1813.

² This *Journal*, iv, p. 91.

³ Liebig's *Ann. d. Chem.*, ccxi, p. 355.

peroxide.¹ The nitrophenylhydrazone from both specimens crystallized from alcohol in yellow needles melting at 91°-92°.

Analysis: 0.1070 gm. gave 0.2277 gm. CO₂ and 0.0586 gm. H₂O.

	Calculated for C ₁₀ H ₁₃ N ₃ O ₂ :	Found:
C.	58.0 per cent.	58.0 per cent.
H.	6.3 " "	6.1 " "

Isobutyric aldehyde, was obtained from the oxidation of isobutyl alcohol with chromic acid and also by the oxidation of α-amido-isovaleric acid with hydrogen peroxide.² The paranitrophenylhydrazone crystallized in orange-yellow needles from alcohol.

Analysis: 0.1390 gm. gave 0.2947 gm. CO₂ and 0.0776 gm. H₂O.

	Calculated for C ₁₀ H ₉ N ₃ O ₂ :	Found:
C.	58.0 per cent.	57.9 per cent.
H.	6.3 " "	6.2 " "

Isovaleric aldehyde was prepared by the oxidation of iso-amyl alcohol with chromic acid and by the oxidation of leucin with hydrogen peroxide.³ The paranitrophenylhydrazone crystallized from alcohol in needles.

Analysis: 0.1137 gm. gave 0.2493 gm. CO₂ and 0.0711 gm. H₂O.

	Calculated for C ₁₁ H ₁₅ N ₃ O ₂ :	Found:
C.	59.7 per cent.	59.8 per cent.
H.	6.8 " "	6.9 " "

Glyoxylic acid. The acid employed was a specimen kindly presented by the firm of Kinzelberger of Prague and was prepared by the electrolytic reduction of oxalic acid. The melting point of this compound was not very sharp as it appeared to undergo some decomposition when slowly heated to near its point of fusion. It crystallizes from alcohol, in which it is but sparingly soluble, in curious nodular aggregates of platelets. On long standing the substance appears to pass into a more insoluble modification.

¹ This *Journal*, iv, p. 63.

² *Ibid.*

³ *Ibid.*

Analysis: 0.1290 gm. gave 0.2160 gm. CO_2 and 0.0410 gm. H_2O

	Calculated for $\text{C}_8\text{H}_7\text{N}_3\text{O}_4$:	Found:
C.....	45.9 per cent.	45.7 per cent.
H.....	3.35 " "	3.5 " "

Acetone. The paranitrophenylhydrazone of acetone has already been described by Bamberger and Sternitzki. It crystallizes from alcohol in long yellow needles and melts at 149° . The formation of this substance constitutes perhaps the best method for the satisfactory identification of acetone. The test may be successfully applied to distillates containing only a few milligrams of acetone and the compound is readily obtained in a state of purity after recrystallization from alcohol.

Methylethyl ketone. The paranitrophenylhydrazone of this substance resembles the corresponding acetone derivatives in every respect. It crystallizes in long yellow needles and is readily soluble in hot alcohol.

Analysis: 0.1138 gm. gave 0.2420 gm. CO_2 and 0.0655 gm. H_2O .

	Calculated for $\text{C}_{10}\text{H}_{13}\text{N}_3\text{O}_2$:	Found:
C.....	57.9 per cent.	58.0 per cent.
H.....	6.3 " "	6.4 " "

Methylisopropyl ketone. The nitrophenylhydrazone of this substance closely resembles the preceding one, but is slightly more soluble in alcohol. It crystallizes in long glistening, orange-yellow needles.

Analysis: 0.1005 gm. gave 0.2211 gm. CO_2 and 0.0624 gm. H_2O .

	Calculated for $\text{C}_{11}\text{H}_{15}\text{N}_3\text{O}_2$:	Found:
C.....	59.7 per cent.	60.0 per cent.
H.....	6.8 " "	6.9 " "

Methyl-n-nonyl ketone. This substance readily forms a crystalline paranitrophenylhydrazone. It crystallizes from alcohol in yellow needles, melting at 90° – 91° .

Analysis: 0.1122 gm. gave 0.2754 gm. CO_2 and 0.0915 gm. H_2O .

	Calculated for $\text{C}_{17}\text{H}_{27}\text{N}_3\text{O}_2$:	Found:
C.....	66.9 per cent.	66.9 per cent.
H.....	8.9 " "	9.1 " "

THE RELATION OF NITRIFYING BACTERIA TO THE UROROSEIN REACTION OF NENCKI AND SIEBER.

By C. A. HERTER. .

(Received for publication January 7, 1908).

In 1892 Nencki and Sieber¹ found that when pure hydrochloric acid was added to the urine of a diabetic patient under their observation, there resulted a beautiful rose-red color. They were sufficiently interested in this little discovery to study the cause of the rose-red coloration, and although they did not succeed in obtaining in a chemically pure state the substance concerned in this reaction, they at least established its most important characteristics, thus rendering it easy for others to recognize its presence. In the course of their studies Nencki and Sieber noted the presence of urorosein (as they called their new coloring matter) in the course of a variety of diseases, such as osteomalacia, nephritis, typhoid fever, carcinoma of the œsophagus, ulcer of the stomach and perityphlitis. Although they obtained well-marked reactions for the first time from the urine of a diabetic patient, they failed to elicit it in some other instances of this disease. They state that the coloring-matter was found in about 10 per cent of the pathological urines examined by them. On the other hand, they failed to find it in the urine of healthy individuals.

The following are among the chief features noted by Nencki and Sieber in respect to their urorosein. The rose color was developed in the cold by the addition of sulphuric or hydrochloric acid. The coloring substance so induced was found to pass readily into amyl alcohol. The spectroscopic examination of the red amyl alcohol solution showed the presence of a characteristic absorption band in the green portion of the spectrum, its location being somewhat nearer the sodium line D than the line E. Only mineral acids were observed to bring out the reaction, acetic acid, for example, being unable to induce it.

¹ *Journ. f. prakt. Chem.*, xxvi, p. 333, 1882.

Nencki and Sieber state that their urorosein differs distinctly from urobilin and indigo blue and they further maintain that it differs from coloring matters observed previously in pathological urines. They emphasize especially the fact that the red coloring matter described by Plosz¹ is wholly different from their urorosein. On the other hand they point out certain resemblances to the rosaniline dyes, especially to fuchsin, which in very dilute solutions not only gives the same nuance as urorosein but also gives a similar absorption band, but one somewhat nearer the violet. They observed also that commercial acid fuchsin in alcoholic solutions shows an absorption band, the position of which is exactly that of urorosein—a point to be noted in this connection as illustrating the fact that two dyes constitutionally different, even according to the view of Nencki and Sieber, may have the same spectrum.

At the conclusion of their paper Nencki and Sieber suggest the possibility that their urorosein arises from a mother substance formed in the intestinal canal as a decomposition product of protein through some form of bacterial organism of not very common occurrence. They suggest further that an aromatic base, isomeric aldehyde collidin, obtained through the decomposition of gelatin and proteid through putrefaction with pancreas, may arise during intestinal putrefaction and constitute the mother substance of urorosein.² Nencki and Sieber mention that Gautier, who also obtained this base from the decomposition of fish muscle, looked upon it as the ptomaine of Selmi. The authors also expressed confidence that direct feeding experiments with the ptomaines would show whether this view is correct or not. Evidently they failed to institute such experiments.

Finally it was concluded by Nencki and Sieber that in the substance called urorosein we have a new type of urinary coloring matter differing on the one hand from those coloring matters

¹ "Ueber einen neuen krystallinischen farbigen Harnbestandtheil." *Zeitschr. f. physiol. Chem.*, vi, p. 504, 1882.

² Aldehyde collidin fails to give any color reaction when treated with potassium nitrite in the presence of strong hydrochloric acid. It therefore cannot be the mother substance of urorosein, for reasons which will be made evident in this paper.

that are derived from bile pigment and on the other from those that have their origin in skatol and indol.

The most important recent work on urorosein has been done by Rosin,¹ who found that the pigment is present in small quantities in every normal urine. On this point Garrod and Hopkins differ from Rosin for they say that the coloring matter occurs only occasionally under normal conditions. Rosin observed the reaction to be especially active in persons suffering from pulmonary phthisis while Garrod² frequently found it in the urine of chlorotic patients. It is noteworthy that Rosin observed the coloring matter in greater quantities in persons on a vegetable diet than in those on a meat diet, and also found that the urine of horses contains considerable of the coloring matter, though less than that of cattle.³ Rosin furthermore succeeded in crystallizing a chromogen of urorosein by precipitating the concentrated alcoholic solution (obtained by treatment with lead acetate) with ether. This chromogen occurred in colorless, transparent needles which readily dissolved in alcohol and water but not in ether or chloroform. It was imperfectly precipitated by lead acetate as a lead salt soluble in alcohol. The watery or alcoholic solutions as well as the crystals themselves develop a red color in contact with a mineral acid and an oxidizing agent. The pure substance on the addition of hydrochloric acid and barium chloride gives no separation of barium sulphate and can therefore not be regarded as an ethereal sulphate. According to Garrod and Hopkins the chromogen is largely thrown out by saturating the urine with ammonium sulphate—an observation which I can confirm. The alcoholic extract of the precipitate is made red by acid and then shows together with the urorosein band a weak urobilin absorption band. A separation of the chromogen is possible if one only adds enough ammonium sulphate to

¹ "Ueber das Indigoroth (Indirubin)" Virchow's *Archiv. f. path. Anat. u. Physiol.*, cxxiii, p. 519, 1891; "Ein Beitrag zur Lehre von den Harnfarbstoffen (Ueber das sogenannte Urorosein, Harnrosen)," *Deutsch. med. Wochenschr.*, xix, p. 519, 1893.

² Garrod and Hopkins. "On Urobilin. Part 1, The Unity of Urobilin" *Journ. of Physiol.*, xx, p. 112, 1896. Also Garrod: "The Spectroscopic Examination of Urine," *Edinburgh Med. Journ.* n. s., ii, p. 103, 1897.

³ In observations on the urines of two horses I failed to obtain the reaction even on addition of nitrites.

the urine to cause cloudiness, this cloudiness being dependent on the chromogen itself and the urobilin remaining in solution.

I have lately had an opportunity to study a urine showing the urorosein reaction of Nencki and Sieber in a marked degree. This urine was obtained from a child seven years of age suffering from a peculiar variety of intestinal decomposition due to an abnormal type of intestinal flora. The child was very much retarded in skeletal and muscular development but was mentally alert, although undeveloped. A noteworthy peculiarity of the condition was marked protrusion of the abdomen, due presumably to long standing distension of the intestine with gases. This case was brought to my notice by Dr. L. E. Holt and is similar in type to a number of cases occurring in children which I have studied, though less carefully than the present case, in conjunction with Dr. Holt. I desire to report here certain observations upon the urorosein reaction as it was met with in this case, believing that the facts developed in the present instance are such as to place the urorosein reaction of Nencki and Sieber in a different light from that in which they regarded it.

It was observed that samples of the urine from the patient just mentioned, when treated with concentrated hydrochloric acid, frequently developed a brilliant and beautiful rose-red color which faded after some hours. It was found that the coloring matter thus developed corresponded in all essential particulars to the urorosein described by Nencki and Sieber, especially in respect to its solubilities, its behavior with reducing agents and oxidizing agents, and the position of the spectroscopic absorption band observed in amyl alcohol solutions. It was soon observed, however, that in one essential respect the color phenomenon observed in this urine did not coincide with the description of these authors. It was found that the urorosein reaction in our case developed only in such urines as had stood in the laboratory at least twelve or twenty-four hours. Fresh urine from the same patient under no circumstances gave the reaction. The onset of the reaction coincided with the development of a turbidity in the urine due to the presence of bacteria, and suggested a possible bacterial origin for the urorosein reaction. This view was confirmed by experiments directed to this point. It was found that if a fresh urine from our patient was inoculated with bacteria

from the urine which had already developed the urorosein phenomenon, the color reaction was obtainable in from twelve to twenty-four hours, the urine remaining in the laboratory during this period. On the other hand, the control urines which were carefully protected from contamination failed to develop the reaction.

The organisms responsible for this change in the urine were easily obtained in pure culture by plating on bierwort agar plates. They occurred as small, Gram-negative bacilli from 0.75 to 1.5μ in length and from 0.5 to 0.7μ in width. They were usually elliptical or ovoidal in shape, younger forms occurring as diplo-cocco-bacilli or even as diplococci. Some of these coccal forms did not attain a diameter greater than 0.3μ . These organisms produced a moderate amount of gas in sugar bouillon. They did not coagulate milk. They possessed one characteristic which is of special importance in the present connection, namely, their capacity to form nitrites. It was found that sterile urines developed nitrites after inoculation with a pure culture of these bacteria. I have not had an opportunity to compare this nitrifying organism with recognized types of nitrifying bacteria.

In the course of conversation with Dr. Dakin regarding the urorosein phenomenon he suggested the possibility that the bacteria which were evidently concerned in the development of the urorosein reaction might be operative through their ability to form nitrites or nitrates. Experiments showed that this is indeed the case. It was observed that the urine of our patient did not develop the urorosein reaction until nitrites had been developed in it as the result of bacterial action. The presence of nitrites was easily shown by the test with metaphenylendiamin and also by the reaction of Tromssdorff, which depends on the liberation of hydriodic acid from potassium iodide in the presence of starch and zinc chloride, on the addition of dilute sulphuric acid to a urine which contains nitrites. In confirmation of the existence of such a relationship between the presence of nitrites and the development of the urorosein reaction, it was found that in fresh urines from our patient which gave absolutely no color reaction with strong hydrochloric acid, the addition of a few drops of a two-tenths per cent solution of sodium nitrite to the contents of the test-tube regularly and rapidly determined the development

of a urorosein reaction which was in every respect typical. There is thus no doubt that so far as our case is concerned the development of the urorosein reaction is contingent upon the presence of nitrites in the urine.

It was thought possible that the presence of nitrates might exert an effect on the development of the urorosein reaction similar to that exerted in the case of the nitrites. It was found, however, that the urine was free from nitrates. Moreover, although it was found that by adding sodium nitrate to the urine there could be obtained evidence of the urorosein reaction when strong sulphuric acid was added, the color reaction was obtained with such difficulty and so imperfectly as to make it entirely clear that the presence of nitrates would not suffice to explain a reaction such as was regularly obtainable from the urine under consideration.

Although the urorosein reaction was so easily obtained from the fresh urine in our case through the addition of potassium nitrite, it was impossible to obtain the reaction, except as a very faint suggestion from any of the normal urines which were examined by a similar procedure. Evidently, therefore, two distinctive features contribute to determine the formation of the so-called urorosein—one, the liberation of nitrous acid, the other the presence of some chromogen peculiar to the urine in question, or at least existing in it in a far larger amount than in ordinary normal urines. The question of the nature of the urorosein reaction thus hinges on the understanding of the influence of each of these factors.

The relation of the nitrites to the development of Nencki and Sieber's reaction is apparently simple and depends on an oxidation of the mother substance or chromogen of the urorosein. There is no reason to think that the influence of the nitrite depends on the introduction of the nitroso group into the molecule of the mother substance, as in the case of the nitroso-indol reaction. The reason for believing this reaction to depend on simple oxidation is that a color closely resembling urorosein is obtainable through the oxidizing action of a number of different substances, as, for example, potassium permanganate, potassium nitrate, potassium persulphate and chloride of lime. In each case, however, there is a strong tendency to over-oxidation and in consequence of this the urorosein color never develops in full

degree throughout the test-tube, but is limited both in the extent of its appearance and in its duration. The oxidation of the mother pigment by nitrous acid is thus much to be preferred to that of other oxidizing agents, such as chlorine or nitric or sulphuric acids. But even with potassium nitrite some precautions must be taken to guard against over-oxidation on the one hand and under-oxidation on the other. Experience shows that it is best to add strong hydrochloric acid to the urine before adding the nitrite solution, the action of which can thus be carefully controlled. On the other hand, if the nitrites be added to the urine before the addition of strong hydrochloric acid, the maximal color reaction is liable not to be obtained owing either to excessive oxidation or to under-oxidation. In routine work it is probably best to employ an equal volume of strong hydrochloric acid in making this test, although frequently a very marked reaction may be brought out by using a smaller proportion of the acid.

It deserves to be noted that the urochrome color reaction does not always develop in a uniform manner. For example it was found that the urine from our patient sometimes gave with hydrochloric acid and potassium nitrite a purple or violet coloration rather than the typical rose-red color. It was at first thought that this violet color might be due to the formation of indigo, as it was otherwise difficult to explain. It was, however, noticed that this reaction could sometimes be obtained from a urine presumably containing no indoxyl-potassium-sulphate, if one may judge by the inability to obtain any evidence of indigo by means of the ordinary reagents, such as Obermeyer's. It was later observed in this connection that the variation in color between the typical rose-red and the violet depended wholly upon the mode of oxidation with potassium nitrite. This was very clearly shown in instances where through cautious addition of nitrite the typical rose-red color was developed in the upper portion of the test-tube, while the violet color appeared in the lower part, where oxidation was less active, all transitional tints being observed between these two levels. Finally, it may be said in this connection that the violet coloring matter just mentioned differs from indigo in not being removed by chloroform.

As regards the mother substance which constitutes the basis of the urochrome reaction, it is not possible to state its chemical

nature at the present time, the substance being present in such small quantities as to render it difficult to isolate in sufficient amounts to determine its constitution. The fact that preparations of urobilin furnished by Schuchardt gave a typical though not very strong urorosein reaction with potassium nitrite and hydrochloric acid made it necessary to exclude the possibility that urobilin is itself concerned with the reaction. This is especially true because the chloroform extracts obtained from the acidified urine were none of them free from urobilin. It was noticed, however, that the urorosein reaction obtainable from chloroform extracts, containing only a small quantity of urobilin, was far more intense and permanent than the reaction obtained from Schuchardt's urobilin in a concentration in which the latter gave rise to much more deeply colored solutions. It was indeed subsequently found that by following the method described by Rosin the urine could be entirely freed from coloring matters through precipitation first by lead acetate and afterwards by ammonium hydroxide. The filtrate obtained after such precipitations still gave almost as strong a urorosein reaction as the original urine. This constitutes definite evidence that the mother substance of urorosein is not a urinary coloring matter—a result in accord with the observation of Rosin that a small quantity of crystalline, colorless substance represents the mother substance from a large volume of urine. It is worth mentioning that the colorless filtrate just referred to, from which so strong a urorosein reaction was obtained, gave a green color reaction with Ehrlich's paradimethylamidobenzaldehyde and a pronounced red with Millon's reagent. That the latter was not due to phenol was made evident in our case by the fact that the reaction with Millon's reagent still persisted (though much weakened) after the distillation of such phenol as was present in the urine. It therefore seems not impossible that these reactions with Ehrlich's aldehyde and Millon's reagent are dependent upon the mother substance itself. I have at least been unable to purify the substance to such a point as to dissociate these reactions from the urorosein reaction itself.

Although the urorosein reaction is described as evanescent, I was able to obtain from the chloroform extract of the urine under study a coloring-matter of the typical urorosein nuance and of

great intensity, which remained for days with very little diminution in intensity. It is evident, therefore, that in the absence of ordinary urinary constituents the urochrome coloring matter possesses a far greater stability than that which has been ascribed to it.

Among the writers who have dealt with the subject of the urochrome reaction there is at the present time a difference of opinion as to whether this reaction bears any relation to the coloring matter known as the skatol red of the urine. For example, Rossler¹ maintains that skatol red and urochrome are wholly unrelated. Rossler experimented on human beings and obtained from the urine, after the administration of skatol by the mouth, a coloring matter which he regarded as wholly distinct from skatol. On the other hand in a recent paper by Porcher and Hervieux,² it is stated with much confidence that urochrome and skatol red are identical substances. They base this contention mainly on the fact that the skatol red which appears after the administration of skatol to a dog, gives the same typical spectroscopic band, located between D and E, that is obtained when the urochrome pigment is subjected to spectroscopic examination.

I have given some attention to the question of the relationship between skatol red and urochrome and have reached the conclusion that in the case which I have had an opportunity to study there is no relationship between the urochrome coloring matter which may be extracted with amyl alcohol and the skatol red coloring-matter which may be similarly extracted. It is, indeed, true that these coloring matters, which in their amyl alcohol solutions are very similar in the quality of their color, give spectroscopic appearance which may be indistinguishable. From both it is possible to obtain the well defined line between D and E which was first described by Nencki and Sieber for urochrome and which is now claimed to be identical with the band produced by skatol red in the experiments of Porcher and Hervieux. It is, however, true that those coloring matters which present a great similarity

¹ "Ueber Skatolroth und ähnliche Harnfarbstoffe." *Centr. j. inn. Med.*, xxii, p. 847, 1901.

² "Untersuchungen über das Skatol." *Zeitschr. f. physiol. Chem.*, xlv, p. 486.

in their nuances are liable to show close similarity in regard to their spectroscopic bands, without this being actual evidence of identity. A good illustrative instance may be cited from the original paper of Nencki and Sieber in which they observed that acid fuchsin—a pararosanilin dye which gives a closely similar color to that of urorosein—also gives the same spectroscopic band. Yet Nencki and Sieber did not claim that urorosein and pararosanilin sulphonic acid are identical in chemical constitution because of the similarity of their spectroscopic behavior. Thus we may say in respect to the controversy over the relation between urorosein and skatol red that this must be settled on other evidence than the coincidence of the spectral bands from these two coloring matters. There are three features of difference between urorosein and skatol red which appear to me significant and even decisive in the discussion of this question. In the first place the two coloring matters are different in respect to their nuances and in regard to their solubilities. The rose tint of urorosein is lighter, brighter and more purplish than the red of skatol red. Urorosein is readily soluble in water but insoluble in ether and chloroform; skatol red is little soluble in water but somewhat soluble in ether and chloroform. In the second place, if one administers to a dog a dose of skatol, by mouth or subcutaneously, the urine will later contain skatol red, that is, a coloring matter which may be developed by the addition of strong hydrochloric acid to the urine. The urine does not, however, under these circumstances contain urorosein. I have not found it possible by the addition of strong hydrochloric acid and potassium nitrite (or any other oxidizing agent) to develop a coloring matter resembling urorosein in its nuance and its solubilities. Finally in the case which has formed the subject of the present study, I have been unable to obtain from the feces the slightest indication of the presence of skatol, notwithstanding that a large number of examinations have been made with a view to this end. The objection might be made to this argument that such skatol as had been formed in the intestine might have been absorbed from the upper part of the colon or from the small intestine and hence could not come within the range of observation. In reply to this, however, it may be said that on two occasions the contents of the bowel were largely and rapidly

evacuated and that even under these conditions not a trace of skatol was obtainable, although the usual intensity of the urorosein reaction remained apparently unchanged during the period corresponding to this cartharsis. At least, therefore, in the case under observation it may be claimed with fairness that the urorosein reaction has nothing whatever to do with the absorption of skatol from the intestinal tract. Nor do I think any serious contention can be made that skatol could have been formed in the cells during intermediary metabolism.

As regards a possible intestinal origin for the urorosein reaction it is necessary at present to speak with much caution. I have succeeded in obtaining through the action of the intestinal bacteria from the case under observation a coloring matter which appears to have the chief characteristics of urorosein. This coloring-matter was obtained by subjecting sterilized whole milk to the action of very large numbers of fecal bacteria, among which organisms of the bifidus type were extremely numerous. The bacterial process resulted in active fermentation and considerable solution of coagulated casein but no putrefactive products were present. When milk was similarly inoculated with small numbers of bacteria of the same origin there occurred coagulation of casein with little or no digestion and a not very active fermentative process. Under these conditions the urorosein reaction was not obtainable. In an experiment made with a view to inducing the presence of the urorosein mother substance in the urine, through filling the intestine with the fermented milk which gave the urorosein reaction, no success was obtained.

CONCLUSIONS.

1. The urorosein reaction of Nencki and Sieber sometimes and perhaps always depends on bacteria for its development where the reaction is induced by adding concentrated hydrochloric acid to the urine. From a urine giving this reaction a pure culture may be obtained which is capable of altering a sterile urine so that it in turn will give the typical reaction with hydrochloric acid.
2. The bacteria which assume this role are capable of forming nitrites in a previously sterilized urine and it can be shown

that the urorosein reaction depends on the liberation of nitrous acid by the strong acid which is employed.

3. The nitrites exert their influence on the chromogen as especially appropriate oxidizing agents and probably not through the formation of a nitroso compound. Doubtless the presence of the urorosein chromogen would be frequently detected where it is now overlooked owing to a failure to employ nitrites in making the test.

4. Urorosein is distinct from skatol red and its chromogen occurs quite independently of the absorption of skatol from the intestinal tract.

5. The chemical constitution and physiological or pathological significance of the urorosein chromogen are at present unknown.¹

Note on the Influence of Bacteria upon the Behavior of Urine Containing Indoxyl Potassium Sulphate toward Concentrated Hydrochloric Acid.

On account of the frequency with which observations are made on the indican of the urine it is worth while to record the following facts with respect to the influence of bacteria on the action of strong hydrochloric acid on urines containing indoxyl potassium sulphate. These facts were noted in the course of the preceding study on the urorosein reaction.

When bacteria concerned with acid fermentation were transferred from a urine giving the urorosein reaction to a series of urines from normal persons it was found (as stated above) that none of these urines developed the urorosein reaction. It was, however, observed that when concentrated hydrochloric acid was added to some of these urines they were found to develop a violet tint, whereas control samples that had not been inoculated failed to give this tint or else gave it in much slighter degree. On shaking with chloroform this took on the color of indigo. As this behavior was seen only in cases where the urine could be shown by Obermeyer's reagent to contain some indican, it is likely that the blue coloration of the chloroform is in reality due to indigo. I have not studied the phenomenon sufficiently to be certain as to the part played by the inoculated microorganisms. The presence of nitrites of bacterial origin may lead to a better oxidation of indican in the presence of strong hydrochloric acid than when the latter is alone employed. In some instances it was found that a stronger reaction for indigo could be obtained from a contaminated urine by merely adding to it strong hydrochloric acid than by the use of Obermeyer's reagent. For this reason it is evident that

¹ While this paper was going through the press I was able to obtain conclusive evidence that the urorosein chromogen is in reality indol acetic acid. I shall present this evidence in the next number of this journal.

erroneous conclusions may be reached as to the amount of indican present, if a urine has undergone the type of acid fermentation here observed. As is well known the intensity of the indican reaction given by a urine is usually decreased by alkaline decomposition. I have not before been aware that acid fermentation may lead to error in the opposite direction.

I have not examined the phenomenon closely enough to know whether other bacterial products than nitrites are ever responsible for the occurrences in question.

ON INDOLACETIC ACID AS THE CHROMOGEN OF THE "UROROSEIN" OF THE URINE.

By C. A. HERTER.

(Received for publication, January 25, 1908.)

In the last number of this *Journal* it was stated that the mother substance or chromogen on which the urorosein reaction of Nencki and Sieber depends is still unknown. Since this paper was written I have had an opportunity to make experiments which show conclusively that the chromogen of the urorosein reaction is in reality indolacetic acid. Inasmuch as the nature of this color reaction of the urine has heretofore been involved in much obscurity, it is desirable to record at the present time the facts which show the relation of indolacetic acid to the urorosein reaction.

In the publication just mentioned, it was stated that it was possible to obtain by extraction of the pathological urine a solution free from color which reacted characteristically with hydrochloric acid and potassium nitrite and which further gave color reactions with Ehrlich's paradimethylamidobenzaldehyde and with Millon's reagent. Since then it has been found that not only in these respects but in more important ones the chromogen material contained in the urine extract corresponds in every essential particular to indolacetic acid. The opportunity to make this comparison I owe to the fact that Dr. Dakin was so obliging as to prepare for me a quantity of indolacetic acid. This indolacetic acid was obtained in crystalline form by the method first used by Hopkins and Cole.¹ These investigators showed that when tryptophan is acted upon by *B. coli* under partially aerobic conditions, indolacetic acid is formed in considerable quantity in addition to indol.

¹ Hopkins and Cole: "Contributions to the Chemistry of Proteids," pt. ii, "The Constitution of Tryptophane and the Action of Bacteria upon it," *Journ. of Physiol.*, xxix, p. 451, 1903.

On comparing the properties of solutions of crystalline indolacetic acid with the properties of the crystalline substance obtained from a pathological urine derived from a patient suffering from a peculiar type of intestinal bacterial decomposition, it was found that the two agree in the following respects.

(1) The colorless crystals obtained from the ethereal extract of the urine gave with concentrated hydrochloric acid and potassium nitrite a brilliant rose-red, possessing exactly the same nuance as that yielded by a solution of indolacetic acid. This color is very characteristic and differs distinctly from the color given by nitrosoindol.

(2) It was found that when the coloring matter prepared by the action of nitrous acid and strong hydrochloric acid was extracted in amyl alcohol it gave a spectroscopic absorption band indistinguishable from that yielded by the similarly prepared coloring matter from indolacetic acid. This absorption band is very sharply defined, at least on that edge lying toward the D line, and is located in the green portion of the spectrum.

(3) Both indolacetic acid and the crystalline substance recovered from the urine give the same red color reaction with paradimethylamidobenzaldehyde—a reaction differing from that obtained from indol in being much less sensitive as well as not identical in tint.

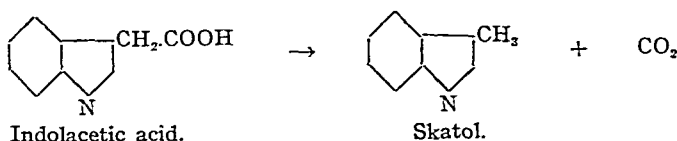
(4) Both substances give the same color reaction with Millon's reagent. As, however, this reagent contains nitrous acid, the significance of this reaction is probably the same as that described in (1). The color obtained is yellow rather than rose-red.

(5) Both substances give the same color reaction with ferric chloride and hydrochloric acid. This is a very delicate and characteristic color reaction which should be carried out with a few drops of a much diluted solution of ferric chloride. The cherry red color develops on heating. This reaction was first described by Salkowski¹ who regarded it as especially satisfactory for the recognition of indolacetic acid.

(6) The most important respect in which there is identity of chemical behavior between indolacetic acid and the substance isolated from the urine is the formation of skatol on heating. If

¹ Salkowski: "Ueber das Verhalten der Skatolcarbonsäure im Organismus" *Zeitschr. f. physiol. Chem.*, ix, p. 23, 1885.

indolacetic acid be heated to a point somewhat above its melting point (melting point, 163° to 164° C.), namely, to about 200° C. the acid loses carbon dioxide and is converted into skatol, in accordance with the following representation:



The operation is best carried out by immersing in a paraffin bath a long, narrow test-tube containing the substance to be tested. The skatol derived from the breakdown of the indolacetic acid condenses on the surface of the test-tube. The upper half of the test-tube is cut off from the lower half and introduced into a larger test-tube. The crystalline material adhering to its inner surface is now extracted with a few cubic centimeters of hot water. The solution thus obtained is tested with paradimethylamidobenzaldehyde in acid solution. On heating a solution containing skatol, to which a few drops of Ehrlich's aldehyde solution has been added, there develops a purple-blue color which deepens on the addition of concentrated hydrochloric acid. On cooling, the blue color gains in intensity. The blue color so obtained passes readily into chloroform. This characteristic reaction for skatol was obtained in its fully developed form from the crystalline substance extracted from the urine.

The foregoing features of agreement between our substance and indolacetic acid suffice to identify the former with the latter. In addition, however, it has been possible to obtain the substance from the urine in a well purified state (recrystallized from benzol) and in sufficient quantity to determine its melting-point, which proved to be 160° to 162° C. There is therefore essential agreement between the known properties of our substance and indolacetic acid, and I have no hesitancy in identifying the uro-rosein mother substance with indolacetic acid.

It is a little singular that the relationship between the uro-rosein of the urine and indolacetic acid should not have been earlier discovered. The publication of Nencki and Sieber in which they first described the uro-rosein reaction was made in 1882. In 1886

Salkowski¹ not only described the properties of indolacetic acid and its formation by bacteria during putrefaction, but also undertook a study of the behavior of the acid in the organism. He described indolacetic acid under the name of skatolcarboxylic acid. Salkowski made a series of observations upon the fate of indolacetic acid in the animal organism. He found that this substance, when administered to rabbits (by stomach) in moderate doses (e. g., 0.4 gm.) found its way to a considerable extent into the urine without undergoing change. He did not succeed in recovering the entire amount given, but this may have depended upon imperfections in the method. That the acid is not burned to any great extent in the organism was shown by the fact that after very moderate doses (e. g., 0.01 gm.) all the characteristic reactions for indolacetic acid could be obtained from the urine of the experimental animal. It would not have been remarkable for Salkowski to have suspected the relation of indolacetic acid to urorosein had he kept in mind the results published by Nencki, nor would it have been surprising had Nencki suspected the nature of his urorosein reaction after reading the observations of Salkowski upon indolacetic acid. Neither writer, however, appears to have concerned himself with the work of the other. Rosin, in 1893, stated that he obtained a small amount of crystalline substance from the urine of patients showing a urorosein reaction, which was capable of yielding certain typical color reactions. He failed however to bring his substance into relation with indolacetic acid.

In the patient from whom was obtained the urine which yielded indolacetic acid, experiment has made it clear that the intestinal tract contains bacteria which are capable of decomposing proteids with the production of indolacetic acid and there can be no doubt that the indolacetic acid of the urine is derived from absorption by the intestinal tract. That this substance is frequently formed to some extent in the course of intestinal putrefaction, was suspected by Salkowski, but was never demonstrated by him or by subsequent writers. I am able to state that the intestinal contents of the patient whom I have had an

¹ *Loc. cit.*; also "Zur Kenntniss der Eiweissfäulniss, II: Die Skatolcarbonsäure, nach gemeinschaftlich mit H. Salkowski in Münster i/W. angestellten Versuchen," *Zeitschr. f. physiol. Chem.*, p. 8.

opportunity to study regularly contains a small quantity of indolacetic acid, sometimes together with indol, sometimes without the latter, and never with skatol.

I hope in the near future to study the occurrence of indolacetic acid in the intestinal tract under pathological conditions¹ and to ascertain the nature of the bacteria which give rise to this decomposition. In the case of marked indolaceturia which I have had under observation one fact regarding the intestinal flora stands out with great clearness. This is the nearly entire absence of the ordinary putrefactive bacteria which lead to the saccharo-butyric form of intestinal putrefaction. The character of the bacteria actually present will be described in another communication. It may be stated here however that the dominant form, *B. bifidus communis* (Tissier) grown on glucose bouillon yielded a substance which gave the characteristic color reaction with potassium nitrite and hydrochloric acid.

In the light of what has been said in this paper it is evident that bacteriologists employing the nitrite reaction for indol should take great care not to confound indolacetic acid with indol. The test for indol should never be made except on the distillate.

¹ Under physiological conditions there may occur a degree of indolaceturia if the subject consumes very large quantities of meat. This fact will be amplified in a subsequent publication.

ADSORPTION OF ACIDS BY CASEIN.

BY LUCIUS L. VAN SLYKE AND DONALD D. VAN SLYKE.

(Received for publication, January 20, 1908.)

In a preceding number of this *Journal*,¹ Dr. T. Brailsford Robertson criticises a conclusion reached by us as a result of our study of the action of dilute acids upon casein² under conditions of low temperature and concentration of acids, such that the solution of the proteid was avoided.

We wish to acknowledge the courtesy of Dr. Robertson in submitting to us in advance of publication a copy of his criticism and also in materially modifying some of his original statements as the result of correspondence between us.

Briefly reviewing the results which formed the basis of the conclusion under criticism, we showed that

(1) Casein takes up a large proportion of the acid from dilute solutions of sulphuric, hydrochloric, lactic and acetic acids without going into solution itself.

(2) The amounts of acid taken up per gram of casein are not constant but vary among other conditions, especially, (a) in proportion to the concentration of the acid in contact with the casein, and (b) with the kind of acid used, there being no definite combining proportions between acid and casein.

(3) The acid taken up by casein is extracted by treatment with water.

In our discussion of the results, it was stated that

(i) The quantitative characteristics of the reaction are completely at variance with those required by the law of mass action, in case the reaction were one incapable of reaching completion because of its reversibility, that is, the hydrolyzibility of the casein-acid salt which would be formed if the reaction were a chemical one, casein acting as a weak base toward the acids.

¹ This *Journal*, iv, p. 35, 1908.

² "The Action of Dilute Acids upon Casein when no Soluble Compounds are Formed," *Amer. Chem. Journ.*, xxxviii, p. 383, 1907.

(ii) The acids do not distribute themselves between the casein and water in accordance with the laws of solution, so that the extraction of acids from aqueous solution can not be regarded as resulting from their partial solution in the solid casein.

(iii) The characteristics of the reaction, when compared with those of known adsorption phenomena, such as adsorption of acids and other substances by charcoal, are found to agree with the characteristics of such phenomena. Hence, it is concluded casein adsorbs acids from dilute aqueous solution.

Dr. Robertson admits the exclusion of "solid solution" as an explanation of the phenomenon, but regards chemical combination as still sharing with adsorption the probability of being the correct explanation.

Before commenting on the criticisms in detail, we wish to call attention to the fact that much of the difference in interpreting our results is a question of definition. Dr. Robertson bases much of his criticism on a definition of the term "adsorption," which differs materially from the clear, specific definition given in our paper, viz: "By adsorption is meant the process whereby a solid substance A in contact with a solution of a dissolved substance B concentrates B upon its surface, withdrawing a portion of B from solution without forming with it a definite chemical compound" (p. 444). This is in accordance with the view of Freundlich and others that adsorption is a process of condensation of adsorbed substance upon the surface of the adsorbent, an example of concentration at the surface of two phases, a purely physical phenomenon.

Instead of accepting the definition of adsorption given by us, as a basis for his criticisms, Dr. Robertson has adopted the interpretation which Hardy¹ gives of van Bemmelen's views, viz: that adsorption is a process on the vague border line between the physical and chemical, resulting from a "mechanical affinity" so closely allied to chemical that adsorption combinations may be regarded as chemical compounds of indefinite composition.

While this use of the term adsorption is accepted by a considerable number of biologists it involves, we believe, an ambiguity that is justifiable on no good ground and calculated only to befog

¹*Journ. of Physiol.*, xxxiii, p. 333.

and mislead. Biological chemists differ among themselves in their misuse of the term adsorption, as we should expect under the circumstances. Adsorption is a phenomenon which has been carefully studied by Freundlich, Losev and others with adsorbents, such as charcoal, absolutely inert, in a chemical sense, towards the adsorbed substances. In such cases, chemical combination is out of the question. It is true, as van Bemmelen points out,¹ that adsorption may at times be "the forerunner of chemical combination," when such combination between adsorbent and adsorbed substance is possible; but the occurrence of powerful adsorption, where chemical combination is impossible, should prevent the confusing view which introduces "loose chemical combination" into the explanation.

In our correspondence with Dr. Robertson, referred to above, he suggests that it would be well to leave "adsorption" as a term to cover "loose chemical combination," to which many biologists are applying it, and to adopt the expression "capillary condensation" to denote the surface action by which chemically inert substances condense others in solution upon themselves. While these distinctions would greatly clarify matters, we believe that "adsorption" has been too long applied to physical reactions comparatively well defined to be dissociated from its connection with these; it has the right of first possession. The same end would be better reached, in our judgment, by devising some other term for indefinite chemical reactions that are not clearly understood. We suggest, in case of those of proteids with acids, bases, etc., combinations which are indefinite but apparently chemical, that we call them frankly "indefinite proteid-base (or proteid-acid) compounds," until a clearer understanding of their nature warrants a more definite nomenclature. Dr. Robertson will probably agree with us in saying that nothing can be gained by wrenching from its familiar environment a term that is capable of meaning something clear and definite and transferring it into a field where it can be used as a convenient dumping ground for a great many things we do not know.

We will now turn our attention to Dr. Robertson's detailed criticisms of our conclusions.

¹ *Zeitschr. f. anorgan. Chem.*, xxiii, p. 342, 1900.

He suggests (p. 36) that our application of the mass law may not have been justified by the conditions. From the fact that casein, itself insoluble in water, can take up acids without being dissolved, we concluded that if a hydrolyzible salt of casein and acid be formed under these conditions, the salt is insoluble. Consequently, of the reacting bodies, casein, acid and acid-casein salt, only the acid is soluble. This being the case, the mass law requires¹ that at equilibrium at a given temperature, the concentration of the free acid be a constant; if acid of greater concentration than this constant were brought into contact with casein, it should be combined until the free acid were reduced to the given concentration; if acid of equal or less concentration than the constant were used, none should be combined. Examples were quoted showing that these conditions are met in chemical reactions of this class, in which but one reagent is soluble. We found that a considerable proportion (40 to 75 per cent) of the acid was always taken up by casein, regardless of how dilute it might be and that there was no such thing as an equilibrium constant.

Dr. Robertson suggests (p. 36) that some casein-acid combination may have been dissolved, thus rendering unjustified our application of the mass law. Inasmuch, however, as we tested the solutions in all cases by a method which we found capable of showing the presence of dissolved casein in 1 to 10,000 dilution, we do not believe that this criticism holds good. In the solution, when cold and dilute, adsorption could become practically complete before solution could be detected.

However, Dr. Robertson suggests further that the proportion of casein acting as a base may have been caused to vary with the concentration of acid in contact with the proteid, referring, in support of this statement, to his results showing such behavior in the case of serum globulin. Granting the correctness of the derivation of his figures on the somewhat difficult subject of proteid isomerization, they do not indicate an effect of the acid approximating in magnitude that found in the change of acid-fixing ability of casein with changing acid concentration.

For example, Dr. Robertson's Table VI² shows that, in one case, decreasing the H^+ concentration to one-sixth (from 72.7

¹ *Amer. Chem. Journ.*, xxxviii, pp. 439-442.

² *Journ. of Phys. Chem.* xi, p. 160.

$\times 10^{-4}$ to 12.4×10^{-4}) decreased the basicity of the globulin only by a very small amount (from 47.38 to 46.68). We found that a similar change in acid concentration from .001958 normal to .0003704 normal sulphuric acid) reduced the acid-fixing power of the casein from 604.2 cc. $\frac{N}{1600}$ normal acid per gram of casein to 262.2; and the effect was even more decided in the case of the other acids.

On the other hand, the change is, quantitatively, of the kind that is characteristic of adsorption. It follows the adsorption formula $\frac{c_1^p}{c_2} = \beta$ (c_1 and c_2 being the concentrations of adsorbed substance in adsorbent and in solution, respectively, at equilibrium; β and p being constants dependent upon the reacting substances, p being usually greater than 1). In the case of adsorption by charcoal, Freundlich found p to be a value generally in the neighborhood of 2. In the case of adsorption of sulphuric acid by casein, it was 1.95. In the case of lactic and hydrochloric acids, it was nearer 1. These acids, in the case of the more concentrated solutions used, dissolved casein slightly, even at low temperature, after several hours of contact, although the greater part of the adsorption was complete before any solution of casein could be detected. Since the equilibrium results, however, were taken from data involving an exposure to contact with acid for twenty-four hours, a noticeable solution of proteid occurred in the more concentrated solutions of hydrochloric and lactic acids;¹ and this solvent action of the more concentrated solutions tended to decrease the value of p to such an extent that it approached 1 more nearly than is usual in cases of adsorption.

(It was found that $\frac{c_1}{c_2}$ is abnormally increased when casein is allowed to dissolve by contact with too concentrated acid, or for too long a time to avoid solution; hence the value of p would be reduced, as this effect does not occur with the more dilute solutions.) Acetic acid, which does not so readily dissolve casein, also showed a value of p but little above 1; but, on account of its smaller conductivity and the smaller amounts adsorbed, the experimental error, which would tend to decrease the values

¹ *Amer. Chem. Journ.*, xxxviii, pp. 415 and 423, Tables VIII and XII.

found for p , was comparatively large. Aside from the fact that p was lower in these instances than is usual in adsorption, all the observed characteristics of the reaction between casein and dilute acids coincide qualitatively and quantitatively with those of undoubted adsorption, such as are observed in the case of charcoal.¹ It seems to us that these criteria of adsorption by their combined presence point very strongly to a case of pure adsorption, though, as Robertson has pointed out and as we had already mentioned in the case of some of them, they may individually be shown, at least qualitatively, by certain classes of chemical reactions. That a chemical reaction should show them all, particularly the slight temperature coefficient and the relations between concentration and amount of acid fixed which are expressed by the exponential formula, would be extraordinary. That a combined physical and chemical reaction should show them, would be less unexpected.

The hypothesis of such a combined physical and chemical reaction Dr. Robertson ingeniously suggests as explaining the case in question. Assuming, first, that the acid dissolves in the solid, granular casein, distributing itself between the casein and the water according to its relative solubilities in casein and water; second, that, after dissolving in the solid, free casein, the acid forms a casein-acid salt with it, the reaction being reversible and the casein-acid salt being dissociable into free casein and acid; and third, that the casein-acid salt, like the acid, is soluble *in the solid free casein*, so that there exists the system,—within the casein (*a*) free casein, (*b*) casein salt and (*c*) free acid and (*d*), outside of the casein in the water solution, also free acid:—the reaction in such a system would show the characteristics observed in the case of casein and dilute acids. The velocity and temperature coefficient would be partially determined by the rate of diffusion of acid from water into casein; and the concentration of free acid in the casein, consequently of the casein salt in the free casein, would vary in proportion to the concentration of the acid in the water outside.

Such a reaction, while theoretically interesting, does not appeal to us as having much probable relation to the realities of

¹ *Amer. Chem. Journ.*, xxxviii, pp. 444-449.

the case in question. It is fundamentally important that a theory should be something more than plausible. For the assumptions involved in such an hypothesis, we know of no experimental data applicable to the case in hand. Against its reasonableness, it may be urged that the action observed by us is too rapid for one which depends upon diffusion into a solid. For example, in a case in which at equilibrium 75.7 per cent of the sulphuric acid in a solution was taken up, there was adsorbed in the first 15 minutes 74.4 per cent. (It should be stated here that in our correspondence, Dr. Robertson says this theory is advanced not as a probable explanation but merely as a possibility overlooked by us.)

Considering that the action of casein upon dilute acids has, qualitatively and quantitatively, the characteristics of adsorption, viz: (1) absence of combining proportions or of evidence of a reversible chemical reaction obeying the mass law; (2) velocity of reaction and time required to reach equilibrium, similar to those in observed adsorptions; (3) rapidity with which adsorbed acid can be washed out until equilibrium is restored; (4) low temperature coefficient; (5) absence of change in appearance, solubilities or other apparent properties of casein such as would be expected if it entered into chemical combination—we still consider adsorption as being by far the most probable explanation of the reaction by which casein takes up acids from dilute aqueous solutions.

We do not consider the acceptance of this explanation as involving the repudiation of the view that proteids combine with acids, nor even that acids combine with casein when they dissolve it. We regard it simply as an example of adsorption, as nearly as it is possible to prove adsorption in any case, due presumably to the physical nature of the solid casein.

In relation to the general question as to whether proteids adsorb acids or combine with them, the action of dilute acids on casein is simply one instance of adsorption, as it appears to us, and of significance in relation to the general question only as such. If, however, it could be proved by further experimental evidence, that, in spite of having the characteristics of adsorption, the reaction is really a case of chemical combination masked by the nature of the reacting substances, then it could hardly be argued hereafter that, mere lack of combining proportions, to say nothing of other adsorption criteria, is a proof of adsorption.

AMPHOTERIC ELECTROLYTES.

By HARALD LUNDÉN

(From the Nobel Institution, Physical Chemistry Laboratory, Stockholm.)

(Received for publication, January 1, 1908.)

DISCUSSION OF THE METHODS FOR CALCULATIONS OF THE DISSOCIATION CONSTANTS.

A comparatively great number of amphoteric electrolytes—i. e., electrolytes, which in a dissociating medium behave both as acids and as bases—have now been investigated, and the fact that many proteins have amphoteric properties has drawn the attention of the biologists to that part of physical chemistry. Objections to the methods of research, hitherto used, have recently been enunciated; it will therefore be of interest to discuss this subject.

In a solution of an amphoteric electrolyte we have the following molecules and ions:¹

Molecules and ions	H ⁺	OH ⁻	HR ⁺	ROH ⁻	HROH	R
Concentrations	<i>a</i>	<i>b</i>	<i>d</i>	<i>c</i>	<i>e</i>	<i>f</i>

$e + f = u$ = concentration of the total unionized substance.

The constants k_a and k_b , defined by the equations,

$$k_a = \frac{a \cdot c}{u} \quad [1]$$

and

$$k_b = \frac{b \cdot d}{u} \quad [2]$$

are called: k_a , the acidic dissociation constant and k_b , the basic dissociation constant. These constants are accessible to measure-

¹ G. Bredig: *Zeitschr. f. Elektrochem.*, vi, p. 33, 1899; J. Walker: *Zeitschr. f. physikal. Chem.*, xlix, p. 82, 1904; li, p. 706, 1905.

ments. The "true" dissociation constants, defined by the equations

$$k'_a = \frac{a \cdot c}{c} \quad [3]$$

and

$$k'_b = \frac{b \cdot d}{c} \quad [4]$$

can not be determined by the methods hitherto used.¹

Two different methods have been adopted for determinations of k_a and k_b .

Method 1. The electrical conductivity of solutions of the pure electrolyte is measured, and from the figures obtained the greater dissociation constant (for example, k_a) can be calculated, if the smaller one (for example, k_b) has been previously determined.² This method is to be used when the greater constant has a value higher than 10^{-7} or 10^{-8} . If the constant is less than 10^{-7} or 10^{-8} , the conductivity is so feeble that it can not be measured with accuracy. In using this method we do *not neglect* one of the constants (for example, the basic) while determining the other.

Method 2. A small dissociation constant (less than 10^{-9} or 10^{-10}) may be calculated from measurements of the hydrolysis of a salt, the chlorid (respectively, the sodium salt) is used, when we wish to determine the basic (respectively, the acidic) constant.³ The calculations are made in absolutely the same way as for common acids or bases. In this case we consequently neglect one of the constants while determining the other.

In a recent paper T. Brailsford Robertson⁴ has raised objections to this method of calculation. According to this author "this method can only give even approximately accurate values

¹ The "true" dissociation constant for ammonia has been determined by T. S. Moore, *Journ. Chem. Soc.*, xci, p. 1382, 1907. (See below.)

² J. Walker: *Zeitschr. f. physikal. Chem.*, xlix, p. 82, 1904; li, p. 706, 1905.

³ For determination of dissociation constants with values between 10^{-7} and 10^{-10} I recommend another method (see H. Lundén, *Journ. chimie phys.*, v, p. 145, 1907; *Meddelanden fr. Vct. Akad. Nobelinstitut*, i, no. 7.

⁴ T. Brailsford Robertson: *Journ. of Physical Chem.*, xi, p. 437, 1907.

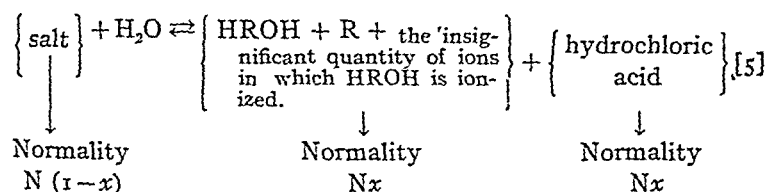
for the larger function if it be sufficiently large compared with the other; otherwise the dissociation constants obtained in this way are subject to considerable error."

I will now show that this method can very well be used even for determining the smaller constant without perceptible error.

Suppose we determine the hydrolysis of the chloride, HRCI , of the amphoteric electrolyte, HROH . Let N be the total concentration (the normality), x the degree of hydrolysis. The solution contains the following molecules and ions:

Molecules and ions	$\left\{ \begin{array}{l} \text{H}^+ \quad \text{OH}^- \quad \text{ROH}^- \quad \text{HR}^+ \quad \text{HROH} \quad \text{R} \quad \text{Cl}^- \quad \text{HCl} \quad \text{HRCI} \end{array} \right.$
Concen- trations	$\left\{ \begin{array}{ccccccc} a & b & c & d & e & f & g \end{array} \right.$
	$\underbrace{e + f = u}$

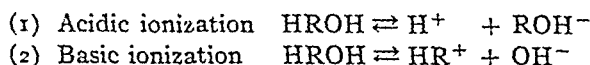
The chlorid is partly hydrolyzed; the hydrolytic equilibrium is the following:



At the dilutions generally used in the investigations, we may without sensible error suppose HRCI and HCl to be completely ionized. Hence:

$$g = N \quad [6]$$

The molecule HROH (which is set free by hydrolytic decomposition) is ionized in two ways:



The product of the concentrations of the ions of water is a constant, or

$$K_w = a \cdot b \quad [7]$$

The solution of the hydrolyzed chlorid contains HCl , the concentration of the H^+ ions is consequently large. Owing to the small value of K_w the concentration of the OH^- ions is very feeble. The above mentioned basic dissociation of the molecule HROH may therefore be neglected. But by the acidic ionization a certain amount of H^+ ions is formed. Let that quantity be δ . This quantity is also equal to the concentration of the ROH^- ions, derived from the same ionization. The hydrochloric acid being a very strong acid in comparison with the amphoteric electrolyte, the dissociation of HCl is practically not affected by the acidic dissociation of the molecule HROH , we have therefore a right to consider the hydrochloric acid as completely ionized.

The concentration of the H^+ ions is therefore

$$a = Nx + \delta \quad [8]$$

and the concentration of the ROH^- ions, the only source of which is the acidic dissociation of the molecule HROH , is

$$c = \delta \quad [9]$$

The concentration of the HR^+ ions (only source: the ionization of the salt HRCl) is:

$$d = N(1 - x) \quad [10]$$

The total concentration is the sum of the substances containing R . Hence:

$$N = c + d + e + f = c + d + u$$

Using the equations [9] and [10], we obtain

$$N = \delta + N - Nx + u$$

or

$$u = Nx - \delta \quad [11]$$

This result may also be obtained in the following manner: A certain amount of the amphoteric electrolyte is set free by the hydrolytic decomposition of the salt (see equation [5]). This amount consists of the molecules HROH and R and of the ions in which HROH is ionized. The concentration of each of these ions is δ . The *normality* (not the osmotic concentration) of the free amphoteric electrolyte is therefore

$$Nx = e + f + \delta = u + \delta$$

hence:

$$u = Nx - \delta$$

According to the definition of the basic dissociation constant (see equation [2]), we have:

$$k_b = \frac{b \cdot d}{u} = \frac{b \cdot N (1 - x)}{Nx - \delta}$$

Using equation [7] we obtain

$$\frac{k_b}{K_w} = \frac{N (1 - x)}{(Nx + \delta) (Nx - \delta)} = \frac{1 - x}{Nx^2} \cdot \frac{1}{1 - \left(\frac{\delta}{Nx}\right)^2} \quad [12]$$

According to the definition of the acidic dissociation constant (see equation [1]), we have:

$$k_a = \frac{a \cdot c}{u} = \frac{(Nx + \delta) \delta}{Nx - \delta} \quad [13]$$

or

$$k_a > \delta \quad [14]$$

For a common base (e. g., ammonia, aniline) the dissociation constant of which is k_b , the equation which expresses the relation between this constant and the hydrolysis of the chlorid is

$$\frac{k_b}{K_w} = \frac{1 - x}{Nx^2} \quad [15]$$

The quantity $1 - \left(\frac{\delta}{Nx}\right)^2$ in equation [12] can therefore be considered as a correction factor and the correction is *increased* if we substitute δ for k_a (see equation [14]). We put therefore

$$\frac{k_b}{K_w} = \frac{1 - x}{Nx^2} \cdot \frac{1}{1 - \left(\frac{k_a}{Nx}\right)^2} \quad [16]$$

If we then can show that the correction factor $1 - \left(\frac{k_a}{Nx}\right)^2$ is

of no importance, it follows that the real correction factor $1 - \left(\frac{\partial}{N\alpha}\right)^2$ is of still less importance.

Table 1 contains the degrees of hydrolysis for common bases (or acids), calculated by equation [15].

From the figures of Table 1 we see that when $k_b < 10^{-9}$ (i.e., for such cases in which determinations of k_b by means of hydrolysis experiments are possible) we have, at the dilutions generally used in the investigations $\left(v = \frac{1}{N} < 100\right)$, $N\alpha > 10^{-4}$. If k_a is less than 10^{-5} , the correction is therefore less than 1 per cent. Such a correction may be neglected. From Table 5 we see that the amphoteric electrolytes hitherto investigated have dissociation constants with such values that the correction factor is of no importance. We may therefore draw this conclusion: *The hydrolysis of salts of the amphoteric electrolytes hitherto investigated has the same value as it has for salts of common bases or acids with the same dissociation constants.*

But from this assertion it is not allowed to draw the conclusion that no errors are introduced, when we use the common method for the calculation of the dissociation constant, because the experimental figures do *not* give us *directly* the degree of hydrolysis.

We will here discuss the two most commonly used methods for determining the hydrolysis.

(1) *The catalytic method.* We measure the rate at which ethyl acetate is catalyzed by a solution of an hydrolyzed salt (for example, a chlorid). That is the same as to measure the concentration of the H^+ ions in the solution. The rate of inversion of cane sugar is also available for the same purpose. For calculation, we use the formula:

$$\frac{k_b}{K_w} = \frac{N - a}{a^2} \quad [17]$$

N = the normality of the salt.

a = the concentration of the H^+ ions.

(2) *The method of Walker-Bredig.* We measure the conductivity of a solution of the hydrolyzed salt (the chlorid or the

TABLE I

k_h K_w or k_h		10^8 10^{-8}	10^5 10^{-5}	10^4 10^{-10}	10^3 10^{-11}	10^2 10^{-12}	10 10^{-13}
N_{∞} 0.1	{ $x =$	0.0032	0.0100	0.0311	0.0955	0.270	0.618
N_{∞} 0.01		0.0100	0.0311	0.0955	0.270	0.618	0.916
N_{∞} 0.001		0.0311	0.0955	0.270	0.618	0.916	0.990
N_{∞} 0.1	{ $Nx =$	3.16×10^{-4}	9.96×10^{-4}	3.11×10^{-3}	9.55×10^{-3}	27.0×10^{-3}	01.8×10^{-2}
N_{∞} 0.01		1.10×10^{-4}	3.11×10^{-4}	9.96×10^{-4}	2.70×10^{-3}	6.18×10^{-3}	9.16×10^{-3}
N_{∞} 0.001		0.31×10^{-4}	0.996×10^{-4}	0.27×10^{-3}	0.62×10^{-3}	0.92×10^{-3}	0.99×10^{-3}
N_{∞} 0.1	{ $N(1-x)$	9.96×10^{-3}	9.9×10^{-3}	9.7×10^{-3}	9.0×10^{-3}	7.3×10^{-3}	3.8×10^{-3}
N_{∞} 0.01		0.99×10^{-3}	0.97×10^{-3}	0.99×10^{-3}	0.73×10^{-3}	0.38×10^{-3}	0.084×10^{-3}
N_{∞} 0.001		0.10×10^{-3}	0.99×10^{-3}	0.07×10^{-3}	0.04×10^{-3}	0.008×10^{-3}	0.001×10^{-3}

sodium salt). Suppose we are dealing with the chlorid of an amphoteric electrolyte. Using the same designations as before we have:

$$\begin{aligned} 10^3 \cdot H = & \underbrace{Nx \cdot \gamma_{HCl} \{A_{\infty} HCl\}}_{\text{conductivity of the hydrochloric acid}} + \underbrace{N(1-x) \cdot \gamma_{\text{salt}} \{A_{\infty} HCl\}}_{\text{conductivity of the salt}} \\ & + \underbrace{\delta \{A_{\infty} ROH\} + \delta \{A_{\infty} H\}}_{\text{conductivity of the amphoteric electrolyte}} \end{aligned}$$

H = specific conductivity.

A = molecular conductivity.

γ = degree of ionization.

$A_{\infty} ROH$ and $A_{\infty} HR$ are both small as compared with $A_{\infty} H$.* We have always

$$\{A_{\infty} ROH\} < \frac{1}{10} \{A_{\infty} H\}; \quad \{A_{\infty} HR\} < \left\{ \frac{1}{10} A_{\infty} H \right\}$$

In the first approximation we may therefore neglect the term $\delta \{A_{\infty} ROH\}$.

$A_1 = 10^3 \cdot H \cdot \frac{1}{N}$ is the apparent molecular conductivity of the salt solution. $A_v = \gamma \cdot A_{\infty}$ is the molecular conductivity at the dilution $v = \frac{1}{N}$.

The ionization of HCl is diminished by the Cl^- ions from the salt HCl , and the ionization of the salt is also diminished by the Cl^- ions from HCl . We put therefore¹

$$\gamma_{HCl} = \gamma_{\text{salt}}$$

or

$$\gamma_{HCl} = \frac{A_v HCl}{A_{\infty} HCl} = \frac{A_v HCl}{A_{\infty} HCl} = \gamma_{\text{salt}}$$

* See W. Nernst: *Theoretische Chemie*, 5te Aufl., p. 388, 1907.

¹ Compare H. Lundén: *Journ. chimie phys.*, v, p, 150, 1907; *Meddelanden fr. Vet. Akad. Nobelinstitut*, i, no. 7, p. 5, 1907.

Then we have:

$$x = \frac{A_1 - \{A_v \text{ HCl}\}}{\{A_v \text{ HCl}\} - \{A_v \text{ HRCI}\}} - \frac{\frac{\delta}{N} \{A_\infty \text{ H}\}}{\{A_v \text{ HCl}\} - \{A_v \text{ HRCI}\}}$$

$\{A_v \text{ HCl}\} - \{A_v - \text{HRCI}\}$ is approximately equal to $\{A_\infty \text{ H}\} - \{A_\infty \text{ HR}\}$

But $A_\infty \text{ HR}$ is small as compared with $A_\infty \text{ H}$. We put therefore:

$$x = \frac{A_1 - \{A_v \text{ HRCI}\}}{\{A_v \text{ HCl}\} - \{A_v \text{ HRCI}\}} - \frac{\delta}{N}$$

Hence:

$$Nx + \delta = N \cdot \frac{A_1 - \{A_v \text{ HRCI}\}}{\{A_v \text{ HCl}\} - \{A_v \text{ HRCI}\}} = a$$

For calculations the formula [17] is used. The experimentally determined quantity is

$$\frac{A_1 - \{A_v \text{ HRCI}\}}{\{A_v \text{ HCl}\} - \{A_v \text{ HRCI}\}}$$

Hence on using these methods (the catalytic method or the method of Walker-Bredig) we obtain at first a value of a (the concentration of the hydrogen ions) and from this value the dissociation constant is calculated.

We will now see, if any errors are introduced by this method of calculation.

For amphoteric electrolytes we obtain by using the equations [8] and [12]:

$$\frac{k_b}{K_w} = \frac{N - a}{a^2} \cdot \frac{1 + \frac{\delta}{N(1-x)} - \delta}{1 - \frac{2\delta}{Nx + \delta}} \quad [18]$$

If we put

$$\frac{k_b}{K_w} = \frac{N-a}{a^2} \cdot \frac{1 + \frac{k_a}{N(1-x) - k_a}}{1 - \frac{2k_a}{Nx}}$$

we increase the correction factor (see equation [14]).

We put therefore:

$$\frac{k_b}{K_w} = \frac{N-a}{a^2} \left\{ 1 + \frac{1}{\frac{N(1-x)}{k_a} - 1} \right\} \left\{ 1 + \frac{2k_a}{Nx} + \left(\frac{2k_a}{Nx} \right)^2 + \left(\frac{2k_a}{Nx} \right)^3 + \dots \right\} \quad [19]$$

$\frac{N-a}{a^2}$ is equal to the constant that is calculated in the common way from equation [17]. The error in $\frac{k_b}{K_w}$ produced in using equation [17] instead of the exact one is consequently less than 1 per cent, if we have:

$$\frac{2k_a}{Nx} \leq 0.005 \quad \text{and} \quad \frac{N(1-x)}{k_a} \geq 200$$

or

$$\frac{2k_a}{Nx} \leq 0.006 \quad \text{and} \quad \frac{N(1-x)}{k_a} \geq 250$$

$$\begin{array}{ccc} \cdot & & \cdot \\ \cdot & & \cdot \\ \cdot & & \cdot \end{array}$$

or

$$\frac{2k_a}{Nx} \leq 0.009 \quad \text{and} \quad \frac{N(1-x)}{k_a} \geq 1000$$

Hence:

For having the error in $\frac{k_b}{K_w}$ less than 1 per cent it is sufficient that k_a does not exceed the values given in Table 2, column 2.

TABLE 2

The constant that is to be determined by measurements of the hydrolysis of a salt.	Allowed limits for the other constant. This constant must not exceed the values:		The degree of hydrolysis of the salt has the values:	
	at $N = 0.1$.	at $N = 0.01$.	at $N = 0.1$.	at $N = 0.01$.
10^{-9}	5×10^{-6}	1.5×10^{-6}	0.010	0.031
10^{-10}	10^{-5}	4×10^{-6}	0.031	0.096
10^{-11}	4×10^{-5}	10^{-5}	0.096	0.270
10^{-12}	10^{-4}	1.5×10^{-5}	0.270	0.618
10^{-13}	1.5×10^{-4}	7×10^{-6}	0.618	0.916

In these cases the error in $\frac{k_b}{K_w}$ is less than 1 per cent if we use the simple formula [17] instead of the true one (formula [18]).

But when the hydrolysis is great a given percentage error in the measured H^+ ion concentration corresponds to a much greater percentage error in $\frac{k_b}{K_w}$. We have (see equation [17]):

$$\frac{d\left(\frac{k_b}{K_w}\right)}{\frac{k_b}{K_w}} = - \frac{2N - a}{N - a} \cdot \frac{da}{a}$$

Assuming an error of 1 per cent in the experimentally determined quantity a , we find the following errors (F) in $\frac{k_b}{K_w}$.

$$N = 0.1 \quad \left\{ \begin{array}{l} a \quad 0.01 \quad 0.02 \quad 0.05 \quad 0.06 \quad 0.08 \quad 0.09 \\ F \quad 2 \quad 2 \quad 3 \quad 3.5 \quad 6 \quad 11 \text{ per cent.} \end{array} \right.$$

$$N = 0.01 \quad \left\{ \begin{array}{l} a \quad 0.001 \quad 0.002 \quad 0.005 \quad 0.006 \quad 0.008 \quad 0.009 \\ F \quad 2 \quad 2 \quad 3 \quad 3.5 \quad 6 \quad 11 \text{ per cent.} \end{array} \right.$$

From these figures we conclude that the correction factors may be neglected when

$$k_a < 2 \times 10^{-4} \text{ for } k_b = 10^{-12} \text{ at } N = 0.1$$

$$k_a < 5 \times 10^{-5} \text{ for } k_b = 10^{-12} \text{ at } N = 0.01$$

$$k_a < 5 \times 10^{-4} \text{ for } k_b = 10^{-13} \text{ at } N = 0.1$$

$$k_a < 8 \times 10^{-5} \text{ for } k_b = 10^{-13} \text{ at } N = 0.01$$

In these cases we may neglect the acidic character of the amphoteric electrolyte, when we determine the basic constant from hydrolysis experiments (for example, from measurements of the hydrolysis of the chlorid). This depends upon the fact that by the hydrolytic decomposition of the chlorid hydrochloric acid is formed, and this strong acid annihilates practically the acidic ionization of the amphoteric electrolyte.

From Table 5 is to be seen that of the amphoteric electrolytes hitherto investigated aspartic acid is the electrolyte for which the correction factors have the greatest value. K. Winkelblech has measured the hydrolysis of the hydrochlorid by the catalytic

method. He has found (from formula [17]) the value $\frac{k_b}{K_w} = 110$.

The dilution used in this experiment was $v = \frac{1}{N} = 10.05$. In

this case the correction factors are of no importance. But Winkelblech has also measured the hydrolysis by means of the method of Walker-Bredig (measurement of the conductivity of the hydrochlorid). In this investigation he has used much greater dilutions, and a correction is therefore necessary. To calculate the correction factors we proceed in the following manner:

The degree of hydrolysis is the same as for a common base with the same dissociation constant (compare Table 1 and equation [12]). Using the value $\frac{k_b}{K_w} = 110$ already obtained, we calculate

Nx and $N(1 - x)$. From equation [13] we find by successive approximations the value of δ . The figures calculated by Winkelblech are equal to $\frac{N - a}{a^2}$. By substituting the values of

Nx , $N(1 - x)$, δ and $\frac{N - a}{a^2}$ in equation [18] we obtain the true

values of $\frac{k_b}{K_w}$. (If the true value $\frac{k_b}{K_w} = 110$ had not been previously known, we must have made successive approximations).

TABLE 3.

$\frac{1}{N}$	VALUES CALCULATED BY WINKELBLECH.		VALUES CALCULATED FROM EQUATIONS [13] AND [18].		
	$\left(\frac{k_b}{K_w}\right)_{\text{Winkelblech}} = \frac{N-a}{a^2}$	x	$\frac{k_b}{K_w}$	x	$\delta \times 10^3$
32	109	0.414	112	0.413	0.15
64	102	0.539	109	0.525	0.15
128	94	0.670	105	0.644	0.14
256	80	0.800	102	0.750	0.14
512	53	0.914	107	0.845	0.13

The corrected figures give fairly concordant results at different dilutions, while the uncorrected figures show wide divergencies, and the corrected values agree with the value obtained by the catalytic method. The figures that Winkelblech has obtained by using the method of Walker-Bredig are perhaps not very accurate. His figures for alanin show also wide divergencies, but in this case the correction factors may be neglected. The simple formula ought therefore to give concordant values at different dilutions. The discrepancies may depend on the fact that by the method of Walker-Bredig impurities have a relatively great influence; further we must know the conductivity of the unhydrolyzed salt with accuracy, but as this conductivity can not be measured directly, it is clear that this circumstance may cause great errors.

It is therefore possible that even the corrected figures in Table 3 are not reliable. But they are of interest, because they indicate the magnitude of errors caused by using the simple formula instead of the true one.

For the other amphoteric electrolytes contained in Table 5 no correction is necessary.

We may now draw the following conclusion:

The dissociation constants of amphoteric electrolytes (for example, the basic dissociation constants) may be calculated from hydrolysis experiments in the same manner as for common bases, provided the acidic dissociation constants are not too great. (Table 2 shows the allowed limits.) When the acidic constants are comparatively great (greater than 10^{-5} or 10^{-6})

the simple formula is not always applicable. In such case we first calculate an approximate value of δ from equation [13], and then we obtain from equation [18] the value of $\frac{k_b}{K_w}$ by successive approximations.

In dealing with proteins such cases (one of the constants greater than 10^{-5}) are not likely to occur.

DISSOCIATION OF SERUM GLOBULIN.

In the above mentioned paper T. Brailsford Robertson has tried to estimate the basic and acidic properties of serum globulin. He investigated solution of globulin in presence of HCl (and KOH) by means of the gas-chain and measured the concentration of the H^+ (and OH^-) ions and he also determined the electrical conductivity of the solutions.

Consider the globulin in presence of HCl

Molecules and ions	$\left\{ \begin{array}{ccccc} & & \text{Protein ions} & & \\ & & \overline{ROH^- \quad HR^+} & & \\ H^+ & OH^- & & Cl^- & \overbrace{HRCI}^{\text{Undissociated salt}} \end{array} \right.$					
Concen- trations	a	b	c	d	γ	u
Ion velocities in $\frac{\text{cm.}}{\text{sec.}}$	U	$-$	v	v	V	$-$

a_1 = the total amount of the acid HCl, which is present combined or uncombined.

λ = the difference between the measured conductivity H of the solution and the calculated conductivity H_1 of the uncombined HCl.

$$m = a_1 - a; \quad H = \frac{V - v}{V + v} \cdot \frac{K_w \cdot k_a}{k_b}.$$

From the equations [16], [13] and [10] in the paper of Robertson it follows that

$$\left\{ \begin{array}{l} a_1 - a = m = \frac{\lambda}{V+v} - \frac{\frac{2v}{V^2-v^2} \cdot H \cdot \lambda}{a^2 - H} = d - c \\ \mu = m - d - c \\ \text{Hence:} \\ \mu = 0 \\ \text{and} \\ \gamma = a + d - c = a + a_1 - a = a_1 \end{array} \right.$$

Robertson consequently makes the supposition that the salt is completely ionized ($\mu = 0$), and he also makes the supposition that the concentration of the Cl^- ion is equal to the total concentration of Cl . ($\gamma = a_1$). These are the same suppositions as I have made in the former part of this paper.

The calculations are executed in the following manner: From the gas-chain experiment he obtains the difference between the total concentration a_1 and the concentration of hydrogen ions a . But on the other hand this quantity is equal to an expression

$$a_1 - a = \frac{\lambda}{V+v} - \frac{\frac{2v}{V^2-v^2} \cdot \lambda \cdot \frac{V-v}{V+v} \cdot \frac{k'_a \cdot K_w}{k'_b}}{a^2 - \frac{V-v}{V+v} \cdot \frac{k'_a \cdot K_w}{k'_b}} \quad [20]$$

which involves the "true" dissociation constants k'_a and k'_b (see equations [3] and [4]). It is easy to show that

$$\frac{k'_a}{k'_b} = \frac{k_a}{k_b}$$

From equation [20] Robertson calculates the quantity $\frac{k_a}{k_b} \cdot K_w$.

It is however possible to make a much simpler calculation. Using the units of Robertson we have the conductivity H (in reciprocal ohms) of the solution containing globulin and HCl :

$$1.037 \times 10^{-2} \cdot H = a_1 \cdot V + a \cdot U + (d + c) v$$

But

$$d + c = 2 d - (d - c) = 2 d - (a_1 - a)$$

Hence:

$$1.037 \times 10^{-2} \cdot H = a_1 (V - v) + a (U + v) + 2dv \quad [21]$$

The values that Robertson has used in the calculations that he has made from the equation [20], are the following:¹

$$V = 65 \times 10^{-5}, v = 7 \times 10^{-5}, U = 316 \times 10^{-5} \frac{\text{cm.}}{\text{sec.}}$$

Using equation [21] and the values of a and H determined by Robertson (see Table I, p. 447, in his paper²) we obtain the figures in Table 4a.

But the figures of Robertson can also be controlled in a quite different way. We can calculate d and c from the equations:

$$\left. \begin{array}{l} \text{Equation [7] in} \\ \text{Robertson's paper}^3 \end{array} \right\} \frac{d}{c} = \frac{a^2 \cdot k_b}{K_w \cdot k_a} \quad [22]$$

$$\left. \begin{array}{l} \text{The same equation as equa-} \\ \text{tion [8] in Robertson's paper} \end{array} \right\} d - c = a_1 - a \quad [23]$$

and from the value

$$K_w \cdot \frac{k_a}{k_b} = 68.3 \times 10^{-5}$$

given by Robertson.

In that manner we obtain the figures in Table 4b.

The values of d and c calculated from equations [21] and [23] are all absurd. In No. 1 c is negative, in Nos. 2, 3, 4, 5 and 6 d is greater than a_1 , or the concentration of one of the protein ions

¹ See the equation on page 446 in the paper of Robertson. The formula [20] does not contain U , but the value that corresponds to the figure $V = 65 \times 10^{-5}$ is the value $U = 316 \times 10^{-5}$ given above.

² In Robertson's paper the specific conductivity H is represented by the letter κ .

³ By a misprint the equation [7] in Robertson's paper is written $d = \frac{a^2 \cdot k_b}{K_w \cdot k_a}$

is greater than the concentration of the Cl^- ion. That is impossible. Alterations in the values of U , V and v do not make the calculation much more reasonable.

TABLE 4a.

TABLE 4b.

Figures calculated from equations [21] and [23].						Values calculated from the value $K_w \cdot \frac{k_a}{k_b} = 68.3 \times 10^{-8}$	
No.	$10^4 \cdot K_w \cdot \frac{k_a}{k_b}$	$a_1 \cdot 10^4$	$a \cdot 10^4$	$d \cdot 10^4$	$c \cdot 10^4$	$d \cdot 10^4$	$c \cdot 10^4$
1	-12	19.3	4.4	9	-6	-5.9	-20.8
2	39	28.9	8.7	41	21	207	187
3	107	38.5	12.4	86	60	47	21
4	340	57.8	23.8	86	52	39	4.7
5	1060	77.0	36.8	185	145	42	2.1
6	4460	120.3	72.7	280	236	48	0.6

Even the figures that are calculated from the value of $K_w \cdot \frac{k_a}{k_b}$ given by Robertson are impossible. In No. 1 d and c are negative, in No. 2 d and c are both greater than the Cl^- ion concentration, in No. 3 d is greater than a_1 . Besides, the values calculated by the two methods do not agree. These impossible results may depend upon the fact that the figures obtained from the gas-chain experiments are not comparable with the conductivity figures. (For example, the impurities could affect the figures in different ways.) But there is also another matter that should be considered. Globulin is not a monovalent base or a monovalent acid. In globulin there exists more than one NH_2 (respectively, COOH) group. And it is not probable that *only one* of these groups should be responsible for the whole basic (respectively, acidic) character. Moreover it is probable that more than one of these groups takes part in salt formation with about the equal amount. The substance may therefore not be considered as a monovalent base (respectively, acid). But the equations we have used in the calculations are deduced for amphoteric electrolytes acting as monovalent bases and monovalent acids. These equations should therefore not be adopted in the case of

globulin. It is also to be noticed that, in dealing with proteins, we do not know the molecular concentrations of the dissolved substance. That involves a new difficulty.

Taking this into consideration we conclude that the formulæ used by Robertson do not represent the chemical equilibriums in globulin solutions. In dealing with proteins it is necessary to take the polyvalent character of these substances into consideration.

Robertson has also tried to estimate the "true" dissociation constants k'_a and k'_b (see equations [3] and [4]) and the molecular concentrations of the unionized globulin molecules; and from these figures he has calculated an approximative value of the molecular weight of globulin. These calculations are however not trustworthy. To show that clearly we will discuss a more simple case.

In solutions of *ammonia* we have two kinds of unionized molecules, as is also the case with amines and amino-acids. Let the molecular concentrations be expressed as follows:

Molecules and ions	$\left\{ \begin{array}{ccccc} \text{H}_4\text{N}^+ & \text{OH}^- & \text{H}_3\text{N} & \text{H}_4\text{NOH} & \text{H}_2\text{O} \end{array} \right.$
Concen- trations	$\left\{ \begin{array}{ccccc} m & m & \underbrace{b \quad c}_{b+c=u} & & d \end{array} \right.$

We have



The dissociation constant, k_b , as determined in the usual way, is defined by the equation:

$$k_b = \frac{m^2}{u} \quad [26]$$

From the equations [24] and [25] we obtain

$$k_b = \frac{k_1 \cdot k_3 \cdot d}{1 + k_3 \cdot d} \quad [27]$$

In solutions not too concentrated the active mass of the solvent water is practically constant. We have therefore:

$$k_3 \cdot d = k_2 \quad [28]$$

$$k_b = \frac{k_1 \cdot k_2}{1 + k_2} \quad [29]$$

From equation [29] it follows that k_b is practically a constant.

For the hydrolysis of ammonium salt (for example, ammonium chlorid) we obtain the same equation as for other bases. Hence:

$$\frac{k_b}{K_w} = \frac{(1-x)}{Nx^2} = \frac{N-a}{a^2} \quad [30]$$

$$\frac{k_1 \cdot k_3 \cdot d}{1 + k_1 \cdot k_3 \cdot d} \cdot \frac{1}{K_w} = \frac{N-a}{a^2} \quad [31]$$

x = the degree of hydrolysis

a = the H^+ ion concentration in the solution of H_4NCl .

Consider solutions of pure ammonia. From conductivity measurement (or from measurement with the gas-chain) we are only able to determine the ion concentrations. We obtain values of m and, as the total concentration is known, also the value of n . Hence, we may calculate the value of k_b from equation [26]. But the only theoretical possibility of determining the "true" constant k_1 by means of conductivity measurements is to use equation [27]. According to this equation k_b is a function of the dilution. From determinations of k_b at different dilutions it is therefore theoretically possible to calculate k_1 and k_3 . (d is a known quantity). But neither the theory nor the experimental methods are exact enough to allow such calculations. At solutions not too concentrated we obtain—within the experimental errors—the same value for k_b at different dilutions, here the experimental methods are not exact enough: at greater concentrations the theory is not exact, because the law of mass action is not applicable in its simple form.

From measurements of the hydrolysis of an ammonium salt, it is also for the same reasons not possible to calculate k_1 and k_3 . We can therefore draw the conclusion:

If we measure the electrical conductivity, the OH^- ion concentration (by the gas-chain or by the catalytic method), the osmotic concentration (by the freezing point method) or the hydrolysis of a salt of the electrolyte in question, we can not from the figures obtained calculate the true dissociation constants for electrolytes (such as ammonia, carbonic acid, amines, amino-acids), in the solutions of which exist different kinds of unionized molecules. Neither are the concentrations of the different unionized molecules obtainable.

If the solution of the electrolyte contains both simple and double molecules—i. e., if the electrolyte is an associating one—we come to the same conclusion. The dissociating constant k_b as obtained in the usual way is theoretically a variable, it is a function of the “true” dissociation constant and of the dilution. By determining the value of k_b at different dilutions it is therefore theoretically possible to calculate the “true” constant. But neither the theory nor the experimental data are exact enough for that purpose.

The above mentioned estimations of Robertson are therefore not reliable.

It is to be noticed that the true dissociation constant can be calculated from measurements of the concentration of one of the unionized molecules. From determinations of the partition coefficient of ammonia between water and chloroform T. S. Moore¹ has calculated the constant for ammonia.

From hydrolysis experiments we obtain the values of $\frac{k_b}{K_w}$ and $\frac{k_a}{K_w}$. From these values k_b and k_a are calculated by assuming the following values of K_w :

	25°	40°
$10^{14} \cdot K_w$	1.11	3.26

For some substances the hydrolysis has been measured by more than one method. In such cases I have considered the figures obtained by the catalytic method as the most reliable. (Compare H. Lunden, *Zeitschr. f. phys. Chemie*, liv, p. 559, 1906.)

¹ T. S. Moore: *Journ. Chem. Soc.*, xci, p. 1382, 1907.

TABLE 5.

Amphoteric Electrolytes for which the Dissociation Constants, both Acidic, k_a , and Basic, k_b , are Known.

	25°		40°	
	k_a	k_b	k_a	k_b
Dimethylpyrone ⁵	0.8×10^{-14}	$2. \times 10^{-14}$		6.6×10^{-14}
Acetoxime ²	6.0×10^{-13}	6.5×10^{-13}	10.2×10^{-13}	19.0×10^{-13}
Heteroxanthin ⁹			4.2×10^{-11}	1.2×10^{-12}
Xanthin ⁹			1.2×10^{-12}	4.8×10^{-14}
Theobromin ⁹	1.1×10^{-10}			4.8×10^{-14}
Methylglycin ⁵	1.2×10^{-10}	1.7×10^{-12}		
Dimethylglycin ⁵	1.3×10^{-10}	9.8×10^{-12}		
Leucin ⁵	1.8×10^{-10}	2.3×10^{-12}		
Glycin ⁵	1.8×10^{-10}	2.7×10^{-12}		
α -Alanin ⁵	1.9×10^{-10}	5.1×10^{-12}		
β -i-Asparagin ⁵	1.35×10^{-9}	1.53×10^{-12}	3.22×10^{-9}	4.23×10^{-12}
Theophyllin ⁹	1.69×10^{-9}	1.9×10^{-14}		5.7×10^{-14}
Dimethyl-o-aminobenzoic acid ¹	2.1×10^{-9}	2.6×10^{-13}		
Histidin ⁴	2.2×10^{-9}	5.7×10^{-9}		
Paraxanthin ⁹	2.3×10^{-9}			3.4×10^{-14}
Phenylalanin ⁴	2.5×10^{-9}	1.3×10^{-12}		
Tyrosin ⁴	$4. \times 10^{-9}$	2.6×10^{-12}		
Leucylglycin ²	1.5×10^{-8}	$3. \times 10^{-11}$		
Alanylglycin ²	1.8×10^{-8}	$2. \times 10^{-11}$		
Glycylglycin ²	1.8×10^{-8}	$2. \times 10^{-11}$		
Cacodylic acid ³	6.4×10^{-7}	3.6×10^{-11}		
Methyl-o-aminobenzoic acid ¹	4.6×10^{-6}	8.55×10^{-13}		
Methyl-m-aminobenzoic acid ¹	$8. \times 10^{-6}$	1.1×10^{-11}		
Dimethyl-m-aminobenzoic acid ¹	$8. \times 10^{-6}$	1.78×10^{-11}		
Methyl-p-aminobenzoic acid ³	9.2×10^{-6}	1.52×10^{-12}		
Dimethyl-p-aminobenzoic acid ³	9.4×10^{-6}	2.99×10^{-12}		
o-Aminobenzoic acid ⁵	1.06×10^{-5}	1.37×10^{-12}	1.35×10^{-5}	3.15×10^{-12}
p-Aminobenzoic acid ⁵	1.21×10^{-5}	2.33×10^{-12}		
m-Aminobenzoic acid ⁵	1.63×10^{-5}	1.22×10^{-11}		
Aspartic acid ⁵	1.5×10^{-4}	1.20×10^{-12}		

Notes to Table 5.

These constants have been determined by the following authors:

¹ A. C. Cumming: *Proceedings of the Roy. Soc., A*, lxxviii, p. 103, 1906. *Zeitschr. f. phys. Chemie*, lvii, p. 574, 1907.

² H. Euler: *Zeitschr. f. physiologische Chemie*, li, p. 219, 1907.

³ J. Johnston: *Ber. d. d. chem. Ges.*, xxxvii, p. 3625, 1904. *Proceedings of the Roy. Soc., A*, lxxvii, p. 82, 1906. *Zeitschr. f. phys. Chemie*, lvii, p. 557, 1907.

⁴ A. Kanitz: *Zeitschr. f. physiologische Chemie*, xlvii, p. 476, 1906. *Pflüger's Archiv*, cxviii, p. 539, 1907.

⁵ H. Lundén: *Arkiv f. Kemi*, Bd. 2, no. 11. *Zeitschr. f. physik. Chemie*, liv, p. 532, 1906.

⁶ P. Walden: *Ber. d. d. chem. Ges.*, xxxiv, p. 4197, 1901.

⁷ J. Walker: *Zeitschr. f. phys. Chemie*, xlix, p. 82, 1904; p. li, p. 706, 1905. *Proceedings of the Roy. Soc.*, lxxiv, pp. 155 and 271, 1904.

⁸ K. Winkelblech: *Zeitschr. f. phys. Chemie*, xxxvi, p. 546, 1901.

⁹ J. K. Wood: *Journ. Chem. Soc.*, lxxxiii, p. 576, 1903; lxxxix, p. 1839, 1906.

SUMMARY.

(1) The hydrolysis of salts of the amphoteric electrolytes hitherto investigated is the same as that of salts of common acids or bases with the same dissociation constants.

(2) The basic dissociation constant of amphoteric electrolytes (or the acidic constant, respectively) may be calculated from hydrolysis experiments in the same way as for common bases (or acids), provided the other constant, the acidic (or the basic, respectively) is not too great. If that is the case, the value of the constant may be obtained by successive approximations (formulae [13] and [18]).

(3) The method of Robertson for determining the basic and acidic properties of globulin is criticised.

(4) A table containing the dissociation constants for the amphoteric electrolytes, hitherto investigated, is given.

ON THE GUANYLIC ACID OF THE SPLEEN.

By WALTER JONES AND L. G. ROWNTREE.

(From the Laboratory of Physiological Chemistry in the Johns Hopkins University.)

(Received for publication, February 19, 1908.)

Ten years ago, Ivar Bang¹ succeeded in isolating from ox pancreas a nucleic acid which differed in a remarkable way from all known substances of this class. According to Bang the compound is characterized by the following properties.

- (1) It contains a pentose group.
- (2) It contains a glycerine group and yields glycerine phosphoric acid. The nucleic acid thus standing as a connecting link between the pentosans and the lecithins establishes a series of physiological chemical relations which can scarcely be found elsewhere in the science.
- (3) It contains an amount of nitrogen and phosphorus relatively greater than is the case with other nucleic acids.
- (4) It yields on hydrolysis an excessive amount of guanin but no trace of either adenin or thymin. On account of this striking difference from other nucleic acids, the substance has received the name of "guanylic acid."

This work which if true would constitute an unmistakable advance in the science has unfortunately been the subject of adverse criticism of a kind from which one would scarcely expect a recovery. Thus v. Fürth,² who has given the subject most careful attention, was able to find among the split products neither glycerine nor a reducing carbohydrate; he claims that the nucleic acid yields adenin as well as guanin and that there is no reason for making any distinction between guanylic acid and thymonucleic acid. In consideration of a number of objections all leading to the same conclusion one might reasonably suppose

¹ Bang: *Zeitschr. f. physiol. Chem.*, xxvi, p. 133; xxxi, p. 411.

² v. Fürth and Jerusalem: *Hofmeister's Beiträge*, x, p. 174.

that v. Fürth's work would finally dispose of the matter of guanylic acid. But Steudel¹ in a very recent communication, takes an entirely different view. He notes that the method of preparation employed by v. Fürth (proposed by Bang and Raaschou²) leads to a nucleic acid of the ordinary type, but that by following the original method of Bang it is possible to obtain from the ox pancreas a true guanylic acid, i. e., an organic compound containing phosphorus which yields guanin but not adenin. Unfortunately Steudel agrees with v. Fürth that guanylic acid contains no glycerine group.

It is the purpose of this communication to show that not only is a true guanylic acid obtainable from ox pancreas but that substances of this class are confined neither to a single animal species nor to a single gland.

THE PREPARATION OF THE NUCLEOPROTEIN OF PIG'S SPLEEN.

Fourteen kilos of carefully trimmed and finely ground fresh tissue were thoroughly mixed in portions with 21 liters of cold distilled water, heated quickly to boiling and the solution filtered off. It is immaterial whether the fluid be filtered hot or after cooling; in either case a fairly clear filtrate is obtained which by repeated filtration through the same filter becomes almost as clear as water but possesses a very pale reddish tinge. If the residue be pressed through linen a milky fluid results which we have not been able to render clearer, either by repeated filtration or by long continued centrifugation; it is therefore advisable to avoid disturbing the residue in any way and to use no greater amount of water for the extraction than that stated. This part of the process was exceedingly more satisfactory than was the case with beef pancreas (see below), where we were never able to obtain anything better than a highly opalescent filtrate, although the solution obtained from this gland is described by various writers as perfectly clear.

The neutral fluid when cool was treated with acetic acid up to 5 to 10 per mille and the precipitated gelatinous nucleoproteid was allowed to subside over night. This nucleoproteid is so

¹ Steudel: *Zeitschr. f. physiol. Chem.*, liii, p. 539.

² Bang and Raaschou: Hofmeister's *Beiträge*, iv, p. 175.

strikingly different in physical properties from the corresponding heavy flocculent substance of ox pancreas that we can scarcely believe the two substances identical. The cloudy fluid was partly decanted and the remainder sharply removed after centrifugation. The nucleoproteid which in this compact form resembles a preparation of starch paste, was freed from soluble impurities by alternate solution in a minimal amount of caustic soda and precipitation with the requisite amount of acetic acid. Suspended matter was removed as far as possible from each alkaline solution by long continued centrifugation, and in the same manner the precipitated nucleoproteid was sharply separated from the supernatant fluid. After this operation had been repeated several times a product was obtained which dissolved in a trace of alkali and on precipitation from the alkaline solution by acetic acid left a perfectly clear fluid. The exceedingly gelatinous nucleoproteid thus purified was dehydrated with alcohol and ether, but unless this is done with the greatest care one will finally obtain a brown sticky mass which is unsuitable for the work that follows. It is necessary to begin with dilute alcohol (50 per cent) and to replace this gradually until absolute alcohol is finally reached. The latter should be repeatedly used and the material allowed to stand for several days in well cooked flasks with frequent and violent agitation. We mention the difficulty of dehydrating this nucleoproteid because we encountered no such difficulty in dealing with the nucleoproteid of ox pancreas and we regard this very striking difference as sufficient ground for assuming that the two nucleoproteids are not identical. This is however entirely aside from the question of the identity of the two nucleic acids. From 14 kilos of moist tissue after the sacrifice of relatively large quantities of material in the interest of a pure product, we finally obtained 64 grams of a perfectly dry pale yellow powder.

THE PREPARATION OF GUANYLIC ACID FROM THE NUCLEOPROTEID OF PIG'S SPLEEN.

The nucleoproteid was treated in portions of 12 grams each with 150 cc. of 2 per cent caustic potash and heated for half an hour in a vessel submerged in boiling water. The red fluid was neutralized with acetic acid and while hot filtered from a small

quantity of perfectly black material; but even after standing 12 hours there was no deposition of guanylic acid although the solution was much more concentrated than that which Bang prepared from pancreas nucleoproteid. (We used only 150 cc. of 2 per cent caustic potash where Bang used 400 cc.) The perfectly clear yellow fluid was then made faintly acid with acetic acid and allowed to stand over night but there was still no deposition of guanylic acid. Several days later when we had concluded that this nucleoproteid yields no substance corresponding to pancreas guanylic acid and after we had practically abandoned the subject, a very small deposit was noticed in the fluid and on the addition of a few drops of acetic acid there was an immediate and copious precipitation of white flocculent material while practically all the coloring matter remained in solution. The precipitate was filtered off, dissolved in hot water and the solution filtered from a small amount of insoluble granular material. On cooling, the pale yellow fluid promptly deposited guanylic acid but on repeating the process the yield soon became noticeably smaller as the acetic acid was removed with the mother liquors. The addition of acetic acid to any of these filtrates causes an immediate precipitation of guanylic acid. This difference in behavior of spleen guanylic acid from pancreas guanylic acid might be explained by differences in the solvent power of the impurities in the two cases but spleen guanylic acid retains this property after purification to such an extent that we are inclined to the opinion that the two nucleic acids are not identical. The original neutral solution generally filters slowly but continuously and as a deposition of guanylic acid is not likely, the slowness of the filtration is without consequence. But it may happen that the neutral fluid cannot be filtered at all. In the one such case which we met, the fluid was markedly acidified with acetic acid, and cooled in ice water when clear and rapid filtration could be made leaving the guanylic acid on the filter. This dark brown residue was boiled with water and a product obtained which was easily filterable and which deposited guanylic acid on cooling. All specimens of what we considered the best products were collected and dissolved in hot water and the guanylic acid which was deposited on cooling the fluid was dried in the ordinary way, with alcohol and ether. From 52 grams of

nucleoproteid after severe losses in the mother liquors for the reason stated we finally obtained 1.58 gram of pure guanylic acid. The substance consists of a perfectly white dry powder soluble in hot water forming a transparent liquid which has an acid reaction to litmus. It responds neither to the biuret nor to Millon's reaction but contains phosphorus and exhibits general properties and reactions which closely accord with those which Bang describes for the guanylic acid of the pancreas.

THE PURIN BASES PRODUCED BY HYDROLYSIS OF THE GUANYLIC
ACID OF PIG'S SPLEEN.

Owing to the misfortune of not knowing in the earlier part of our work that large quantities of guanylic acid may be recovered from mother liquors by the addition of acetic acid, the amount of material at our disposal was insufficient for an exhaustive examination such as we would otherwise have made and as we intend to make in the immediate future; so that we decided to devote all of our material to a final decision of the very important question, whether or not we are here dealing with a true guanylic acid.

A gram and a half of the material was heated for three hours with 25 cc. of 5 per cent sulphuric acid in a vessel submerged in boiling water. On standing over night the fluid deposited in profusion macroscopic needles of guanin sulphate. These were dissolved by warming and the solution was first neutralized and then treated with such an excess of ammonia that the fluid contained 2 per cent of the reagent. The product after digestion in the warm for an hour was allowed to cool and the precipitated guanin filtered off. After thoroughly washing in turn with 1 per cent ammonia and water the base was dissolved in 1 per cent caustic soda and again precipitated by the addition of acetic acid. The precipitate was filtered off, washed, dried and weighed. For the separation of guanin from small quantities of adenin there are two properties of the bases which can be used and can be thoroughly depended upon. First, guanin is almost insoluble in 2 per cent ammonia while adenin dissolves in this reagent with comparative ease. Second, both bases dissolve easily in dilute caustic soda but while guanin is quantitatively precipitated

from such a solution by acetic acid, adenin remains under these conditions completely in solution. It will be noticed that both of these methods were applied in turn to the case which we are describing. The original ammoniacal filtrate from guanin and the acetic acid fluid obtained in its purification were united and treated with silver nitrate and ammonia. The small silver precipitate was thoroughly washed, suspended in boiling water and decomposed with hydrochloric acid. The acid fluid was filtered from silver chloride, evaporated carefully just to dryness and the last traces of hydrochloric acid driven off by moistening with water and again carefully evaporating. The insignificant amount of residue was dissolved in water at 40° and treated with ammonia. A very small precipitate of guanin was formed which showed no inclination to dissolve in ammonia even after the addition of a great excess of the reagent. The fluid was filtered off and boiled until perfectly neutral to litmus. It will be observed that any adenin originally present must now be found in this fluid. Its volume was only 20 cc. yet a portion failed to give a distinct precipitate with silver nitrate and ammonia while in another portion picric acid did not even produce even an opalescence. *The guanylic acid of the spleen gives no trace of adenin.*

The main yield of guanin together with the small amount obtained from the mother liquors weighed 390 milligrams. It was dissolved in hot 5 per cent hydrochloric acid and decolorized with a small amount of animal charcoal. The solution on cooling deposited the characteristic centimeter—long feathery needles of guanin hydrochlorate. The salt was allowed to dry in the air and analyzed with the following results.

1. 0.1742 gram lost 0.0280 gram at 100° and required 7.06 cc. of standard sulphuric acid (1 cc. = 0.0077 gram of nitrogen).

2. 0.1921 gram lost 0.0309 gram at 100° and required 7.85 cc. of the same sulphuric acid.

	Required for $C_5H_5N_5O \cdot HCl \cdot 2H_2O$:	I.	Found: II.
H_2O	16.11 per cent	16.07	16.08 per cent.
N.....	31.33 "	31.21	31.46 "

It may appear that our conclusion, viz: that we are here dealing with a guanylic acid, is based on an experiment with rather a small amount of material. In answer to such an objection we

would state that we should use no more if we were to repeat the work with an unlimited supply of material at our disposal. We are now engaged in an investigation of nucleic acids which involves a large number of just such analyses as that described and where the amount of material is of no consideration to us. Experience in these cases has taught us that the best quantitative results can be obtained by using no more nucleic acid than will produce 300 to 400 milligrams of the base sought.

ON THE DISTRIBUTION OF GUANYLIC ACID IN THE ORGANISM.

We are now occupied with the examination of a number of glands for substances of this type and have uniformly found substances whose physical properties correspond closely with those of guanylic acid. The pig's pancreas yields a perfectly clear aqueous extract from which acetic acid precipitates a flocculent nucleoproteid which closely resembles the nucleoproteid of ox pancreas and from which a guanylic acid can be prepared which cannot be distinguished by any apparent difference from ox pancreas guanylic acid. With ox pancreas we experienced considerable difficulty. The glands used were perfectly fresh and the method given by Bang closely followed but in spite of every effort we were unable to prepare any thing approaching a clear aqueous extract. However, the cloudy fluid gives a nucleoproteid which in turn yields a true guanylic acid.

Our results show conclusively that guanylic acid (or the guanylic acids¹) are considerably more widely distributed than was formerly supposed and lend in great measure to the belief that these substances are common nuclear constituents. As all specimens of ordinary nucleic acid hitherto prepared have been found to yield adenin as well as guanin it seems certain that the glands which yield guanylic acid must also contain either "adenylic acid" or nucleic acids which produce both bases.

Since writing the above article our attention has been called to the work of Odenius² who prepared guanylic acid from the nucleoproteid of the mammary gland.

¹ It is possible that future investigations will demonstrate unmistakable differences among the nucleic acids of this class, thus establishing a series of guanylic acids.

² See Maly's *Jahresbericht*, xxxix, 1900.

THE ISOLATION OF CARNAUBIC ACID FROM BEEF KIDNEY.

By EDWARD K. DUNHAM, M.D.¹

(Received for publication, February 12, 1908.)

The existence, in animal tissues, of saturated fatty acids, higher in the series than stearic acid, has received so little attention that the discovery of such an acid in alcoholic extracts from beef kidney appears worthy of record.

The acid in question is a constituent of a lipoid having solubilities similar to those of Liebreich's "protagon" and possibly related to cerebron. It can readily be obtained from the lipoid by cleavage with either acids or alkalies. The simplest mode of isolation is by cleavage in absolute alcohol containing from 2 to 5 per cent of alcoholic hydrochloric acid, or an equivalent amount of sulphuric acid, at a moderately elevated temperature. On cooling the solution, a mixture of the acid and its ethyl ester separates as a white precipitate. From this mixture the acid may be obtained by saponification with sodium ethylate and freeing the acid from the resulting soap with a mineral acid. Both the free acid and its ethyl ester are very soluble in ether and chloroform and in hot alcohol, benzene, acetone, ethylacetate or acetic acid, but separate from these solvents on cooling. Some ester is readily formed on heating the acid with alcohol. It is, therefore important to avoid this solvent in purifying the acid.

The analyses given below were made on different samples of the acid, isolated from different lots of beef kidneys, showing that the acid is a constant constituent of a substance regularly obtainable from this organ.

¹ The work required for the results here recorded was done in part at the University and Bellevue Hospital Medical College, in part at the Rockefeller Institute for Medical Research.

A. ANALYSES OF THE FREE ACID:

- I. Preparation of October 24, 1907. Purified by slow chilling from 80 per cent alcohol.

Combustion November 14. Substance, 0.1256; CO_2 , 0.3592; H_2O , 0.1474.

C, 77.997 per cent; H, 13.119 per cent.

- II. Preparation of November 8, 1907. Purified by chilling from, first, benzene, second, ethyl acetate.

Combustion December 4. Substance, 0.1331; CO_2 , 0.3809; H_2O , 0.1580.

C, 78.040 per cent; H, 13.307 per cent.

- III. Preparation of November 1, 1907. Purified with acetone, benzene and acetic ether, successively.

Combustion December 26. Substance, 0.1519; CO_2 , 0.4347; H_2O , 0.1776.

C, 78.048 per cent; H, 13.083 per cent.

- IV. Preparation of January 4, 1908. Purified by fractional precipitation with magnesium acetate; decomposing the magnesium soap with hydrochloric acid and recrystallizing the fatty acid from hot acetone.

Combustion January 14. Substance, 0.1590; CO_2 , 0.4556; H_2O , 0.1870.

C, 78.147 per cent; H, 13.161 per cent; O, 8.692 per cent.

Calculated for $\text{C}_{24}\text{H}_{45}\text{O}_2$:

C, 78.159 per cent; H, 13.156 per cent; O, 8.684 per cent.

B. ANALYSIS OF SILVER SALT. From the sample of acid prepared January 4, and, presumably, the most pure, a silver salt was made, of which 0.5294 gram on ignition, yielded 0.1240 gram of residue, or 23.423 per cent. It was found impossible to burn off all the carbon at a moderate temperature. The residue was therefore treated with nitric acid and the silver determined by titration with tenth-normal ammonium sulphocyanate. Of this solution 11.2 cc. gave a distinct end-reaction with the ferric alum used as indicator; corresponding to 22.833 per cent of silver in the sample used for this determination. The theoretical amount of silver in $\text{AgC}_{24}\text{H}_{47}\text{O}_2$ is 22.700 per cent, which would, in this case, be equivalent to 11.14 cc. of the ammonium sulphocyanate solution.

C. ANALYSIS OF THE ETHYL ESTER. The ethyl ester was obtained from a mixture of free acid and ester, resulting from the original cleavage of the lipid, by two successive treatments with absolute alcohol, one-twentieth saturated with hydrochloric acid, on a water-bath with reflux condenser for about five hours. The white precipitate separating from this solution on cooling was purified with acetone twice and dried *in vacuo* over sulphuric acid. It was white, had a silky luster and a consistency of soft paraffin.

Combustion February 12, 1908. Substance, 0.1414; CO_2 , 0.4079; H_2O , 0.1677.

C, 78.670 per cent; H, 13.271 per cent; O, 8.059 per cent.

Calculated for C_2H_5 , $\text{C}_{24}\text{H}_{47}\text{O}_2$:

C, 78.685 per cent; H, 13.245 per cent; O, 8.070 per cent.

The melting point of the acid is 72.4°C ., that of the ethyl ester 50° , on moderately rapid heating. These figures are uncorrected, but that for the acid would require very little correction as but a short thread of mercury projected above the level of the sulphuric acid bath in which thermometer and capillary tube containing the substance were immersed. In both cases the melting points were sharp. The identity of the acid isolated from beef kidneys with the carnaubic acid having the same percentage composition obtained from carnauba wax is made probable by the correspondence in melting points; that of carnaubic acid being 72.5° .

In April, 1905,¹ Thierfelder published work on cerebronic acid, $\text{C}_{25}\text{H}_{50}\text{O}_2$, and its methyl ester on hydrolysis in methyl alcohol containing 10 per cent of sulphuric acid. Cerebronic acid melts at 99° to 100° and has a percentage composition: C, 75.38; H, 12.56; O, 12.06.

The cerebronic acid obtained by Thierfelder is certainly not identical with the renal carnaubic acid, but it is of interest that such high fatty acids should be constituents of lipoids from the brain and kidney that present analogies suggesting similarity in constitution.

¹ *Zeitschr. f. physiol. Chem.*, xliv, p. 366.

THE PEROXIDASE REACTION OF MILK.

BY J. H. KASTLE AND MADISON B. PORCH.

(From the Division of Chemistry, Hygienic Laboratory, U. S. Public Health and Marine Hospital Service, Washington, D. C.)

The peroxidase reaction of milk, which depends upon the fact that small amounts of raw milk induce the oxidation of various leuco compounds by hydrogen peroxide, has been turned to practical account as the most convenient means of distinguishing between raw and boiled milk.¹ A great variety of substances have been employed for this purpose, among which may be mentioned guaiacum, paraphenylene diamine, the potassium iodide-starch reagent, ortol, amidol, ursol D., paradiethylphenylene diamine, and phenolphthalin. According to Franz Utz,² however, the latter compound has not given satisfaction. Indeed, it has been the experience of certain observers that all of these color reagents are more or less unsatisfactory, and that the peroxidase reaction of milk is more or less untrustworthy and unreliable as a means of distinguishing between raw and boiled milk. Other methods depending upon the quantity of soluble nitrogen in the whey have therefore been proposed by Rubner³ and confirmed by Middleton.⁴ Obviously however the determination of the amount of soluble nitrogen in a large number of samples of milk entails considerable time and labor and therefore is practically impossible in the study of the milk supply of a large city, which in order to be of any practical value must necessarily include the examination of a large number of samples

¹ The literature on this subject is very extensive. Those desiring to familiarize themselves with it more at length are referred to a communication by J. H. Kastle and Norman Roberts, entitled "The Chemistry of Milk." See Article 10, Bulletin No. 41, Hygienic Laboratory, "Milk and its Relation to the Public Health."

² *Milch. Zeit.*, xxxii, p. 722, 1903.

³ *Hygien. Rund.*, v, pp. 1021-1022, 1895.

⁴ *Ibid.*, xi, p. 601, 1901.

daily. In an investigation of the Washington milk supply recently carried out in the Hygienic Laboratory, we have had occasion to study the peroxidase reaction of milk, with the view of distinguishing between raw milk and that which had been sterilized by heat.

In the course of our studies on the peroxidase reaction of a number of samples of freshly drawn milk from the same herd we were impressed with the fact that the milks showed great differences in the intensity of the peroxidase reaction towards three of these peroxidase reagents. That such is the case is evident from the results given in Tables I to IV. In these tables a strong, decided peroxidase reaction is marked +, a faint or doubtful reaction, \pm and no reaction, -.

TABLE I. PEROXIDASE REACTION OF RAW MILK.

(Towards Phenolphthalin and Guaiacum.)

November 15, 1907.

Number of sample.	Phenolphthalin.	Guaiacum.
1.....	+	+
2.....	+	+
3.....	+	\pm
4.....	+	+
5.....	\pm	+
6.....	-	-
7.....	\pm	\pm
8.....	\pm	+
9.....	-	+
10.....	+	+
11.....	+	+
12.....	\pm	+
13.....	-	+
14.....	\pm	-
15.....	\pm	-
16.....	\pm	+
17.....	\pm	+
18.....	-	\pm
19.....	-	-
20.....	+	+
21.....	+	+
22.....	-	\pm
23.....	-	\pm
24.....	+	\pm
25.....	\pm	-
26.....	\pm	\pm
27.....	\pm	+
28.....	\pm	+

TABLE II. PEROXIDASE REACTION OF RAW MILK.
(Towards Phenolphthalin, Guaiacum and Paraphenylene diamine.)
November 18, 1907.

Number of sample.	Phenolphthalin.	Guaiacum.	Paraphenylene diamine.
1.....	—	—	+
2.....	—	+	+
3.....	+	+	±
4.....	±	+	+
5.....	—	±	+
6.....	—	+	+
7.....	±	±	+
8.....	—	+	+
9.....	—	—	+
10.....	+	+	+
11.....	+	±	+
12.....	+	±	+
13.....	—	—	+
14.....	+	+	+
15.....	+	—	+
16.....	—	—	+
17.....	—	+	+
18.....	—	—	+
19.....	—	—	+
20.....	±	±	+
21.....	±	+	+
22.....	—	—	+
23.....	±	—	±
24.....	±	+	+
25.....	—	—	±
26.....	+	+	+
27.....	±	+	+
28.....	±	—	+

TABLE III. PEROXIDASE REACTION OF RAW MILK.
(Towards Phenolphthalin, Guaiacum and Paraphenylene diamine.)
November 22, 1907.

Number of sample.	Phenolphthalin.	Guaiacum.	Paraphenylene diamine.
1.....	—	+	—
2.....	+	+	±
3.....	+	+	+
4.....	±	+	+
5.....	—	+	+
6.....	—	±	±
7.....	—	+	±
8.....	—	±	+
9.....	—	+	±
10.....	—	+	+
11.....	±	+	+
12.....	±	+	+
13.....	—	±	+

TABLE IV. PEROXIDASE REACTION OF RAW MILK.

(Towards Phenolphthalin, Guaiacum and Paraphenylen diamine.)
November 27, 1907.

Number of sample.	Phenolphthalin.	Guaiacum.	Paraphenylen diamine.
1.....	+	+	+
2.....	±	+	+
3.....	+	+	+
4.....	+	+	+
5.....	+	+	+
6.....	+	+	+
7.....	±	+	+
8.....	+	+	+
9.....	+	+	+
10.....	—	—	±
11.....	±	+	+
12.....	+	+	+
13.....	—	±	±
14.....	+	+	+

In the scope of the present communication it is practically impossible to enter into any discussion of the variations in color exhibited by different milks towards these peroxidase reagents. Suffice it to say in this connection that in case of the phenolphthalin the colors given by different milks varied from faint pink to deep purplish red; in case of the guaiacum, from light to dark indigo blue; and with the paraphenylen diamine, from a faint pink to a deep rich purple or a dirty shade of blue. In all of our tables, as in Table I, good decided reactions are marked +, faint or doubtful reactions, ± and no reaction, that is, no development of color, —.

The individual milks of the whole or a part of this herd of cows were tested for peroxidase by means of these three reagents, on nine different days during the latter part of November with results similar to those given in Tables I to IV. The results of these tests with phenolphthalin, guaiacum and paraphenylen diamine are given in Table V.

It is evident from the results given in Table V that with paraphenylen diamine 100 specimens of milk out of a total of 135 gave a good peroxidase reaction, 33 gave a faint reaction, and 2 showed no reaction. With guaiacum, 89 specimens out of 135 gave a good peroxidase reaction, 23 a faint reaction, and 23 showed no reaction at all. With phenolphthalin, 49 speci-

mens out of 135 gave a good peroxidase reaction, 33 a faint reaction, and 53 no reaction.

As the peroxidase test is ordinarily carried out there can be no question but that for milk paraphenylene diamine is the most delicate and reliable of the three reagents examined. This conclusion is borne out by the results reached by other investigators. The fact however that it failed to give the reaction with two perfectly fresh, raw milks, out of 135 samples and gave only a faint or doubtful reaction in 33 specimens out of 135, indicates that even this compound is far from being a perfectly safe and reliable reagent for distinguishing between raw and heated milk.

TABLE V. PEROXIDASE REACTION OF MILK.

(Towards Phenolphthalin, Guaiacum and Paraphenylene diamine.)

Date, 1907.	Number of samples.	Phenolphthalin.			Guaiacum.			Paraphenylene diamine.		
		+	±	—	+	±	—	+	±	—
Nov. 18.....	28	7	8	13	12	5	11	26	2	0
" 19.....	13	4	4	5	5	4	3	11	2	0
" 20.....	13	4	3	6	4	5	4	10	3	0
" 22.....	13	4	1	8	10	3	0	8	4	1
" 23.....	12	3	2	7	11	1	0	6	6	0
" 25.....	14	7	4	3	10	2	2	8	5	1
" 26.....	14	6	3	5	10	2	2	8	6	0
" 27.....	14	9	3	2	12	1	1	12	2	0
" 29.....	14	5	5	4	14	0	0	11	3	0
Total.....	135	49	33	53	89	23	23	100	33	2

The phenols as peroxidase reagents and their influence on the oxidation of leuco compounds by hydrogen peroxide in the presence of raw milk. During investigations on the oxidases and the peroxidases extending over several years, one of the authors of the present communication has experienced the need of a suitable antiseptic in order to eliminate the possibility of bacterial action in such oxidations. Unfortunately, the selection of such an antiseptic involves considerable difficulties, for the reason that many substances which have the requisite antiseptic properties interfere with the peroxidase reaction. In our work on the peroxidase reaction of milk, this need has again manifested itself, for in all such work with milk it is particularly desirable

to be able to positively exclude the action of bacteria. During recent years, an antiseptic sold under the tradename of "trikresol" has been used quite extensively by the bacteriologists in the Hygienic Laboratory. This substance is a mixture of the three cresols in approximately equal proportions.

Ordinarily, trikresol is employed as an antiseptic in its 1 per cent aqueous solution. The effect of such a solution on milk has been found to greatly intensify the peroxidase activity of the latter towards guaiacum, phenolphthalin, and paraphenylene diamine, in addition to functioning as an antiseptic. Milks which in their natural state give only weak colorations with these reagents, give brilliant colors with all of these reagents when a 1 per cent solution of trikresol is added to them in the proportion of one-sixth to one-third of the volume of the milk; and specimens of cow's milk or human milk, which ordinarily exhibit no peroxidase reaction, give always in the case of cow's milk, and not infrequently in the case of woman's milk, strong peroxidase reactions. A dilute solution of trikresol therefore acts as a sensitizing agent in the peroxidase reaction of milk.

In this connection, the following observations are of interest as throwing light on this sensitizing action:

When 1 cc. of a 1 per cent solution of trikresol is added to a small amount of cow's milk, 2 cc. to 5 cc., containing from 0.1 cc. to 0.3 cc. of $\frac{M}{10}$ hydrogen peroxide,¹ the mixture at once develops a slight, though unmistakable, yellow color. Tubes containing from 2 cc. to 5 cc. of milk, 1 cc. of 1 per cent trikresol solution and small amounts of hydrogen peroxide, present an altogether different appearance from those containing the same amounts of milk and hydrogen peroxide but containing 1 cc. of distilled water in place of the trikresol solution. On the other hand, when trikresol and hydrogen peroxide are added to milks

¹ During the period covered by this investigation, extending over two months, the same solution of hydrogen peroxide was employed. At the beginning of the investigation this solution was $\frac{M}{10}$. At the conclusion of the investigation it was found to be approximately $\frac{M}{20}$, so that the concentration of the solution employed really ranged between $\frac{M}{10}$ and $\frac{M}{20}$. This variation in concentration is without significance so far as our results and conclusions are concerned. In what follows therefore we shall continue to speak of it as a $\frac{M}{10}$ solution of hydrogen peroxide.

which have been boiled or even heated to 80° C. for 10 to 20 minutes and cooled, no such alteration in color is observable. It is probable, therefore, that under the influence of raw milk the cresols themselves are oxidized by the hydrogen peroxide, and that it is the first product of their oxidation, probably some organic peroxide, or quinoid compound, which accomplishes the oxidation of the leuco compound when such is present. In consequence of the fact that the products of the oxidation of the cresols by peroxidase and hydrogen peroxide are colored compounds, the cresols themselves are delicate peroxidase reagents and doubtless might be employed to advantage in distinguishing between raw milk and that which has been sterilized at a temperature of 80° C. or higher.

The following observations are also of interest in this connection. It has already been pointed out that heated milk, even in the presence of trikresol, fails to show the peroxidase reaction. This of course is in harmony with our present conceptions of the peroxidases. The question suggested itself in this connection however: What would be the effect of heat on a raw milk containing small amounts of hydrogen peroxide and trikresol; and the further question, What would be the effect of heat on a raw milk containing small amounts of hydrogen peroxide, trikresol, and phenolphthalin? In order to answer these questions, the following experiments were carried out:

SERIES I. Four tubes were prepared, each containing the following substances: 5 cc. fresh cow's milk, 0.3 cc. $\frac{N}{10}$ hydrogen peroxide, and 1 cc. of 1 per cent trikresol solution. These tubes were numbered 1, 2, 3 and 4. Tubes 3 and 4 were then heated to 80° C. for 20 minutes, when they were removed from the bath and cooled. Tubes 1 and 2 were kept at room temperature. To each tube 1 cc. of a phenolphthalin solution containing 0.00032 gram of the compound in the form of the neutral sodium salt, and 2 cc. of $\frac{N}{10}$ sodium hydroxide were then added. Tubes 1 and 2 developed the characteristic deep pink color of phenolphthalein in alkali at once, whereas tubes 3 and 4 remained white. All the tubes were distinctly yellowish at first, but tubes 3 and 4 lost their yellow color on being heated.

In order to answer the second question, tubes were prepared containing 5 cc. of fresh cow's milk, and all the reagents essential

for the phenolphthalin peroxidase test, viz: 0.3 cc. $\frac{N}{10}$ hydrogen peroxide, 1 cc. of a 1 per cent trikresol solution, and 1 cc. of the solution of phenolphthalin. The tubes were numbered 5, 6, 7 and 8. Tubes 7 and 8 were heated for 20 minutes at 80° C. and were then cooled to room temperature. To each of the tubes 2 cc. $\frac{N}{10}$ sodium hydroxide were then added. All the tubes showed a deep pink color, 5 and 6 being equal in tint but somewhat lighter in color than the pair of tubes which had been heated.

Finally, four tubes were prepared containing all of the reagents necessary for the phenolphthalin-peroxidase test, even including the alkali, viz: 5 cc. of fresh cow's milk, 1 cc. of a 1 per cent trikresol solution, 0.3 cc. $\frac{N}{10}$ hydrogen peroxide, 1 cc. of phenolphthalin, and 2 cc. $\frac{N}{10}$ sodium hydroxide. The tubes were numbered 9, 10, 11 and 12. Tubes 9 and 10 were allowed to stand 20 minutes at room temperature. Tubes 11 and 12 were heated for 20 minutes at 80° C. and then cooled to room temperature. All of the tubes became deep pink in color. The tubes which were heated to 80° C., namely, 11 and 12, at first developed the color much more rapidly and in greater intensity than the tubes left standing at room temperature. The colors produced in the heated tubes then faded somewhat, so that at the conclusion of the experiment they were decidedly lighter in color than those which had been left at ordinary temperature. It would seem that the results arrived at in these experiments could be satisfactorily explained in the following manner, at any rate, the following explanation affords a good working hypothesis. In the first place, as already pointed out in the above, it would seem that when an aqueous solution of trikresol is added to raw milk containing small amounts of hydrogen peroxide the trikresol is converted into a faintly colored compound, which in the presence of raw milk is capable of oxidizing various leuco compounds more rapidly and effectively than hydrogen peroxide under like conditions. When heated to 80° C. for 20 minutes, in contact with milk and small amounts of hydrogen peroxide, this compound is decomposed. Hence when the phenolphthalin is added it is not oxidized. On the other hand, when the phenolphthalin is added to raw milks containing trikresol and small amounts of hydrogen peroxide and the tubes then heated to 80° C. for 20 minutes it, the phenolphthalin, is oxidized in greater amounts

than in the cold, for the reason that it finds itself in contact with the oxidizing substance produced from the trikresol, the latter oxidizing the phenolphthalin before it, the product of the oxidation of the trikresol, has been destroyed by the heat, and oxidizing it more rapidly than in the cold for the reason that, like all oxidation processes, the one under consideration is accelerated by heat. Similarly when alkali is present, the oxidation of phenolphthalin by the initial product of the oxidation of trikresol by raw milk and hydrogen peroxide is accelerated, and the gradual fading of the color of the resulting phenolphthalein is doubtless due to the action of the alkali which, as is well known, gradually destroys the color of phenolphthalein under certain conditions, as already pointed out by Kastle and Amoss¹ and other observers.

It will be observed therefore that the mixture of raw milk, hydrogen peroxide and trikresol really presents many analogies in conduct to an extract containing an oxidase. It has the power of oxidizing certain leuco compounds and loses its oxidizing activity at 80° C. Indeed, this analogy is so striking that if a chemist were presented with an extract containing a peroxidase, small amounts of hydrogen peroxide and trikresol and undertook to examine it for ferments, he would undoubtedly arrive at the conclusion that the liquid under investigation contained an oxidase. We hope to consider this particular phase of the subject more at length in a subsequent communication.

The effect of other phenols and of a few other organic substances on the peroxidase reaction of milk has also been studied, among which may be mentioned phenol, thymol, phloroglucinol, α and β -naphthol, pyrocatechin, resorcin, hydroquinone, pyrogallol, guaiacol, eugenol, benzoic and salicylic acids, etc. Without entering into the details of the experiments it may be observed that of the substances above named the following were found to give characteristic color reactions with raw milk and hydrogen peroxide:

Name of substance.	Color produced by raw milk and hydrogen peroxide.
Phenol.....	Deep yellow, changing to brownish yellow.
α -Naphthol.....	Blue.
Guaiacol.....	Reddish brown.
Pyrocatechin.....	Violet brown.
Hydroquinone.....	Salmon pink.
Pyrogallol.....	Yellow.

¹ See "Variation in the Peroxidase Activity of the Blood in Health and Disease," by J. H. Kastle and H. L. Amoss. Bulletin No. 31, Hygienic Laboratory.

In control experiments which differed from the original only in that the milk used had previously been boiled and then allowed to cool no changes of color with any of these reagents were observed. It is evident therefore that all of the last named substances might themselves be used advantageously as peroxidase reagents for milk. In fact, one of them, namely, guaiacol,¹ has already been recommended for this purpose.

Of the above-named substances whose effect on the peroxidase reaction of milk has been studied, phenol and β -naphthol were found to greatly accelerate the oxidation of phenolphthalin by raw milk and hydrogen peroxide, while the others were found to be practically without effect in this respect. In the case of certain of these substances, however, it is obviously difficult to positively determine whether they accelerate the peroxidase reaction of milk or not, for the reason that, as already pointed out, certain of them alone give marked colorations with raw milk and hydrogen peroxide. The striking fact nevertheless remains that phenol, the three cresols and β -naphthol greatly intensify the peroxidase reaction of milk, in all probability through the formation of intermediate, unstable compounds possessing the properties of peroxides.

The cresols as an aid to the peroxidase reaction of milk in distinguishing between raw milk and that which has been sterilized by heat. Since our observation that trikresol greatly augments the power of raw milk to induce the oxidation of leuco compounds through the agency of hydrogen peroxide we have tested the peroxidase activity of 120 samples of raw milk, of which 74 were obtained directly from the herd and 46 purchased by the Health Department of the District of Columbia in the open market. The peroxidase activity of these milks was tested towards guaiacum, phenolphthalin, and paraphenylene diamine, using 5 cc. of milk, 1 cc. of a 1 per cent solution of trikresol, 0.3 cc. $\frac{M}{10}$ hydrogen peroxide and from 0.1 cc. to 0.5 cc. or 1 cc. of the peroxidase reagent; and with all of the 120 samples positive peroxidase reactions were obtained; whereas with the same samples heated to 80° C. for 20 minutes, no reaction for peroxidase was obtained with any of these milks. It would seem

¹ *Milch. Zeit.*, xxxii, pp. 594-595, 1903.

therefore that by means of this modified peroxidase reaction we have a ready and simple means of certainly distinguishing between raw milk and that which has been sterilized by heat at a temperature of 80° C. or higher. In fact, as may be seen from the results of our observations given in the next section of this communication, it is possible, in the majority of cases at least, by means of the phenolphthalin-trikresol-hydrogen peroxide reagent, to distinguish between raw milk and that which has been sterilized by heating to 70° C. for short intervals. This method also has the advantage that the tests can be made rapidly and that only small amounts of milk and of the several reagents are required.

On the effect of heat on the peroxidase activity of milk. Aside from its practical application in enabling us to readily distinguish between raw milk and that which has been sterilized by heat at a temperature of 75° C. or higher, the peroxidase reaction of milk is of importance as throwing light on the extent to which changes in the biological properties of milk may be effected by pasteurization at different temperatures. As is well known to those who have given this subject their attention, milk is no longer regarded as an inert fluid, but rather as a secretion possessing well-marked and characteristic biological activities. It may prove the medium of conveying a toxin, and the milk of animals immunized against certain infections has been found to contain the specific antitoxins of these infections. It shows Bordet's reaction, viz: that by the repeated injection of small amounts of milk of a different species into the blood of an animal, the blood serum of the animal so treated acquires the property of curdling the milk of the species whose milk has been injected, somewhat after the manner of rennin. Human milk, in contradistinction to cow's milk, also shows Moro's reaction, in that it has the power of curdling hydrocele fluid. Milk has also been found to contain a number of soluble ferments, among which may be mentioned galactase, amylase, lipase, lacto-kinase, etc. Following the line of thought first suggested by Escherich, certain observers, among whom may be mentioned Marfan,¹ Moro, Engel, Hippius and others have been led to attach considerable importance to the

¹ *La presse medicale*, Paris, 1901, p. 13.

milk ferments and are inclined to look upon them as stimulators and aids to the digestive processes in infants during the period of milk-feeding. According to these investigators, the disturbances of nutrition in milk-fed children are probably due, in part at least, to the absence either in whole or in part, as the result of sterilization by heat or otherwise, of the soluble ferments normally present in raw milk. During recent years therefore in the pasteurization of milk, the tendency has been to reduce the temperature at which the pasteurization is accomplished to the lowest point at which the bacteria, especially the pathogenic forms, are killed; and while at the Nathan Straus milk-depots in New York a temperature of 75° C. is employed, Freeman¹ has recently recommended a temperature of 60° C. for 40 minutes as that best suited for the pasteurization of milk, and still more recently, Rosenau,² working in the Hygienic Laboratory, has observed that an exposure of milk to 60° C. for 20 minutes is sufficient for all ordinary purposes of sterilization and that at this temperature all pathogenic organisms, even including the tubercle bacillus, are either destroyed or rendered inert after an exposure of 20 minutes.

In the light of these facts and in view of what has already been said concerning the possible significance of the milk enzymes for infant nutrition, it becomes a matter of considerable importance to determine the effect of temperature on the peroxidase activity of milk, and especially the effect of heating milk to 60° C. for various lengths of time. Generally speaking, the destruction of a soluble ferment by heat is a function of time and temperature. The higher the temperature the shorter the time-interval required for its destruction, the lower the temperature the longer the time required for its destruction. In the case of certain ferments, notably in the case of rennin, the destruction of the ferment by heat has been found to obey the law of a monomolecular process for temperatures higher than 44° C. A great many less exact observations have been made on the thermal death-point of lacto-

¹ *Journ. of the Amer. Med. Association*, xlix, No. 21, pp. 1740-1742, 1907.

² Bulletin No. 41, Hygienic Laboratory. Article 20—Pasteurization, pp. 589-624. Also Bulletin No. 42, Hygienic Laboratory, Thermal Death-Points of Pathogenic Micro-organisms in Milk. (In press.)

peroxidase. Taken collectively, the greater number of these observations would seem to indicate that a short exposure of milk to 80° C. is sufficient to destroy the peroxidase of milk. Some observers have noted its destruction even at lower temperatures, for example, in the neighborhood of 75° C. Our own results would seem to indicate that no cow's milk which has been heated to 80° C. or even 75° C. for 20 minutes shows any noteworthy peroxidase activity, and that an hour's exposure of a milk to 70° C. is sufficient to destroy the peroxidase.

The peroxidase activity of 13 samples of milk was determined by means of the phenolphthalin-trikresol-hydrogen-peroxide reagent on the raw milks and after heating to 70° C. for 20 minutes. Ten of the samples showed greater peroxidase activity in the raw state; two, greater peroxidase activity after heating to 70° C. for 20 minutes; and with one of the samples, the peroxidase activity was the same before and after heating. It is evident therefore that heating to 70° C., even for comparatively short intervals, is somewhat destructive to the peroxidase of milk. The effect of heating to 60° C. for 20 and 40 minutes, on the peroxidase activity of a large number of samples of milk, some of which were obtained directly from the herd and some from milk dealers in the District of Columbia, was also determined and comparisons made as to the relative peroxidase activity of the raw and heated milks. Out of 100 samples thus compared as to the peroxidase activity, by the phenolphthalin-trikresol-hydrogen-peroxide test, 46 of the samples showed greater peroxidase activity after heating to 60° C. for 20 minutes than in the raw state, 39 of the samples showed greater peroxidase activity in the raw state, and with 15 of the samples the peroxidase activity was the same before and after heating to 60° C. for 20 minutes.

On the other hand, out of 47 samples compared as to peroxidase activity in the raw state and after heating to 60° C. for 40 minutes, 18 of the raw samples showed the greater peroxidase activity; in 15 of the samples heated to 60° C. for 40 minutes it was greater than with the raw milks, and in 14 of the samples the peroxidase activity was the same before and after heating. It is evident therefore from these results that milk sustains no loss in peroxidase activity by heating to 60° C. for short intervals of time. Indeed, in approximately one-half of the samples exam-

ined it was found to slightly, though perceptibly, increase as the result of heating the milk to 60° C. for 20 minutes; and with the milks which had been heated to 60° C. for 40 minutes approximately the same number of samples showed on the one hand an increase, on the other hand a diminution, in peroxidase activity, and in approximately the same number of samples the peroxidase activity remained the same after as before the heating.

The peroxidase activity of human milk. During recent years certain authors, especially Nordmann,¹ have been inclined to attach considerable importance to the peroxidase activity of human milk, and considerable differences of opinion prevail among different observers regarding the significance of the lacto-peroxidase reaction for the nutrition of breast-fed infants. Nordmann is inclined to regard the failure on the part of a human milk to show the peroxidase reaction with Storch's reagent, viz: paraphenylene diamine, as a positive pathological sign of the unfitness of the milk for the nourishment of the child, and is inclined to put this reaction far ahead of the findings of the chemical analysis. In one case investigated by this author a breast-fed infant whose mother's milk showed no peroxidase reaction, suffered from severe colicky pains and the stools showed a fatty diarrhea, which symptoms were promptly relieved by placing the child on a diet consisting of an artificially prepared infant food, supplemented by cow's milk. From his findings in this particular case and from the fact that Kreiss found three specimens of human milk, which had caused no digestive disturbances in breast-fed children, to show the Storch peroxidase reaction, Nordmann arrived at the conclusion that those human milks which fail to show the peroxidase reaction must be regarded as unfit for the nourishment of the child and likely to give rise to digestive disturbances. On the other hand, Thiemich² takes exception to Nordmann's conclusion for the reason that, according to this author, the peroxidase reaction of human milk is very inconstant. He therefore protests against Nordmann's use of this reagent as a test for the lack of tolerance (*Unverträglichkeit*) of a given mother's milk, and against Nordmann's conclusion, viz: that the failure of the Storch reaction with

¹ *Monatsschrift f. Geburtshülfe und Gynaekologie*, xv, pp. 152-158, 1902.

² *Ibid.*, xvi, pp. 32-36.

human milk is to be regarded as an indication that digestive disturbances would likely result from its use as a food for infants. It seemed of interest therefore in this connection to determine the peroxidase activity of as many specimens of human milk as could be obtained, with the view of instituting comparisons between it and cow's milk. Through the kindly coöperation of Dr. H. W. Lawson of the Columbia Hospital of this city we have had at our disposal about six samples of human milk daily for some time, for which, in this connection, we desire to express our thanks and appreciation. The peroxidase activity of these samples has been tested towards guaiacum, phenolphthalin, and paraphenylene diamine, with and without trikresol, as soon as the samples reached the laboratory, with the results given in Table VI. By way of comparison, the peroxidase activity of cow's milk was also determined daily on the milk of a cow that had calved about two weeks before this series of tests was begun. In each test 2 cc. of the milk was employed. To this was added 1 cc. of water or 1 cc. of a 1 per cent solution of trikresol, as required, and 0.1 cc. $\frac{M}{10}$ hydrogen peroxide. To the set of milks tested with guaiacum, 0.5 cc. of a 1 per cent solution of purified guaiacum resin in absolute alcohol or acetone was added in each case and the tubes allowed to stand for a short time before shaking. This we have called the ring test. The tubes were then shaken, after which they were allowed to stand for a longer or shorter time when the color was again noted. To the set of milks tested with phenolphthalin, 1 cc. of an aqueous solution of phenolphthalin, containing 0.00032 gram of the compound in the form of the normal sodium salt, was added in each case, after which the tubes were allowed to stand for 15 minutes at ordinary temperature, when 2 cc. of $\frac{N}{10}$ sodium hydroxide were added to each tube. The color of each tube was then noted for the immediate reaction. The tubes containing an excess of alkali were then allowed to stand over night at ordinary temperature, when the colors were again noted. To the set of milks to be tested with paraphenylene diamine, 0.1 cc. of a saturated alcoholic solution of paraphenylene diamine hydrochloride was added to each sample, allowing the solution to flow over the surface of the milk. The tubes were then allowed to stand undisturbed for a few moments and the colors obtained noted. The

results of the tests as thus carried out are described as the ring test (immediate). The tubes were then shaken and the colors again observed, after which the tubes were allowed to stand over night, when the colors were again noted.

It is evident from the results given in Table VI:

(1) That trikresol greatly intensifies the peroxidase activity of woman's milk or, at least, it enables us to detect some peroxidase activity in those cases in which ordinarily, i. e., in the absence of the cresols, none is revealed by the peroxidase reagents. That such is the case may be seen at a glance by comparing the results given under the heading "Milk + water" with those given under that of "Milk + trikresol." Out of 69 tests on human milk, in the absence of trikresol, only four were positive; and of these, only one was strong and decided. It is interesting to note in this connection that these four positive tests were obtained with paraphenylene diamine. On the other hand, out of a total of 108 tests, with all three reagents in which trikresol was present, 69 were positive and 47 were strong and decided. Of the positive reactions obtained with human milks in the presence of trikresol, guaiacum showed 15, phenolphthalin 24, and paraphenylene diamine 31; and of the strong and decided peroxidase reactions shown by these milks, guaiacum gave 12, phenolphthalin 15, and paraphenylene diamine 21, so that all things considered, paraphenylene diamine seems to be the most delicate of the three peroxidase reagents for human milk.

(2) Of the 39 samples of human milk thus far examined, 34 gave at least some evidence of the peroxidase reaction towards one or all of the three reagents; and with two exceptions, the milks of 23 women whose milk was tested for peroxidase, gave at least some evidence of peroxidase activity during periods of lactation ranging from 3 to 33 days, and in these two exceptional cases, the paraphenylene diamine tests were not allowed to stand over for 24 hours; so that it would seem likely at least that all human milks would give at least some evidence of peroxidase activity towards one or the other or all of these reagents, either immediately or after long standing. As yet our data are too meager to enable us to arrive at any conclusion concerning variations in the peroxidase activity of human milks during the lactation period. We hope to determine this from day to day cover-

TABLE VI. PEROXIDASE REACTION OF HUMAN MILK COMPARED WITH THAT OF COW'S MILK.

(Towards Guaiacum, Phenolphthalin and Paraphenylene diamine, with and without Trikresol.)

Woman (initials).	Date of collection (December, 1907.)	Lactation period (in days).	MILK + WATER.					MILK + TRIKRESOL.				
			Guaiacum.	Phenolphthalin.	Paraphenylene diamine.			Guaiacum.	Phenolphthalin.	Paraphenylene diamine.		
			Immediate (ring test). After shaking. 15 to 30 minutes. Short interval. 24 hours.		Immediate (ring test). After shaking. 24 hours.			Immediate (ring test). After shaking. 15 to 30 minutes. Short interval. 24 hours.		Immediate (ring test). After shaking. 24 hours.		
A. B.	13	10		-	-				-	-		
"	17	14										
"	20	17		-	-	±			±	-	-	+
B. T.	13	8		-	-				±	-	-	±
"	18	13	-	-	-				±	-	±	±
"	20	15	-	-	-				±	-	-	±
M. M.	13	17							±			
R. H.	13	9							±			
"	18	14						+	+	+	+	+
"	20	16	-	-	-			+	+	+	±	+
Ada J. (A. J.)	16	14							±	-	±	±
"	19	17	-	-	-				±	-	±	±
V. N.	16	10							-	-	-	±
N. R.	16	16				±		+	+	±	+	+
M. C.	16	7						±	±	±	±	+
"	20	11		-	-			±	±	±	±	+
B. D.	16	13							+	+	+	+
"	19	16	-	-	-			+	+	+	+	+
F. M.	16	6							+	+	+	+
"	17	7							-	-	+	±
"	20	10	-	-	-				±	-	±	±
A. N.	17	30								±	±	+
"	20	33	-	-	-				+	+	±	±
K. M.	17	4							+	+	+	+
"	21	8		-				+	+	+	+	+
M. R.	17	5			±	+		+	+	+	+	+
L. M.	18	7				±			-	-	±	±
N. Y.	18	17	-	-	-				-	-	±	±
"	21	20							-	-	±	±
L. L.	18	6	-	-	-				±	-	±	+
Hat. W.	19	10	-	-	-				+	+	+	+
V. W.	19	13	-	-	-				-	-	±	±
"	21	15	-	-	-				±	-	-	±
C. M.	18	5	-	-	-			±	+	+	+	+
"	21	8	-	-	-			±	±	+	+	+
A. J.	19	8	-	-	-				-	-	+	+
Maud S.	19	4	-	-	-				±	+	±	+
S. H.	21	3		-	-				-	±	±	±
M. R.	21	6		-	-				-	-	-	-
Cow's (15).	13								+	+		
"	16					±	+	+	+	+	+	+
"	17					±	+	+	+	+	+	+
"	18		±	+	+	±	+	+	+	+	+	+
"	19		±	+	+	±	+	+	+	+	+	+
"	20		±	±	±	±	+	+	+	+	+	+
"	21		±	+	+	±	+	+	+	+	+	+

ing long lactation periods, provided the cases required for this work can be obtained. We hope also to make a study of the peroxidase activity of the milk of different races of women and also of any possible variations in the peroxidase activity of human milk in health and disease. Generally, it has been our experience that human milks exhibiting colostrual characteristics, especially certain highly colored milks, such as the deep orange colored milk of K. M., show the greatest peroxidase activity. And in this connection it is interesting to note that of all specimens of human milk thus far tested, the specimen obtained from K. M., on the eighth day of lactation, is the only human milk which anywhere nearly approached cow's milk in peroxidase activity. This particular specimen marked with a * in Table VI, gave strong peroxidase reactions with all of the reagents, especially in the presence of trikresol.

(3) The striking differences in peroxidase activity between cow's milk and human milk have to be seen in order to be appreciated, and are greatly in favor of the former over the latter; especially is this true where trikresol is employed. It has already been pointed out that since this substance has been employed to intensify the peroxidase reaction of cow's milk, 120 samples, partly from the herd direct and partly from the city market, have been tested; and while they were found to vary a good deal among themselves, all of them, or certainly the great majority of the specimens at least, showed strong peroxidase reactions to each of the reagents, compared with those shown by human milk. That such is the case is evident from the results of the peroxidase tests, on the one cow, given in Table VI. It will be observed that out of a total of 32 peroxidase tests on this cow's milk, 26 were strong and very decided and only one was negative, viz: the ring test with guaiacum in the absence of trikresol, on December 20. The differences in color of the peroxidase tests of cow's milk and human milk are also very striking, especially when trikresol is present. With cow's milk, guaiacum gives a deep indigo blue immediately; phenolphthalin, deep pink to purplish red after 15 minutes; and paraphenylene diamine, a deep rich shade of purple passing into blue almost immediately whereas with woman's milk, all of these colors are of a paler and less pronounced shade; guaiacum giving pale to dark blue, the color

developing slowly; phenolphthalin, traces of pink to deep pink; and paraphenylene diamine, pink to bluish purple, developing more slowly than in the case of cow's milk. Taken collectively, our results point unmistakably to the conclusion that the peroxidase activity of cow's milk is much greater than that of human milk.

The results reached in this investigation lead to the following conclusions:

(1) That raw milks show extreme variability in their power to induce the oxidation of readily oxidizable substances by hydrogen peroxide and that as ordinarily carried out the peroxidase reaction is a very unsafe criterion whereby to determine whether a given specimen of milk is raw or cooked. In general, it may be said that cooked milks, or those which have been sterilized at or above 80°C ., do not show the peroxidase reaction; whereas raw milks, as a class, do give the reaction. The mere fact however that a given specimen of milk does not show the peroxidase reaction as ordinarily carried out, is not, in itself, sufficient to prove that it has been sterilized by heat.

(2) That the power of milk to induce the oxidation of phenolphthalin and other leuco compounds by hydrogen peroxide is greatly intensified by certain substances, among them phenol, the three cresols and β -naphthol; and that by the use of these peroxidase accelerators, phenolphthalin, guaiacum and paraphenylene diamine can all be used to advantage and with certainty as peroxidase reagents for milk; and that by means of the peroxidase reaction thus modified by the use of these sensitizing substances, viz: phenol, the cresols or β -naphthol, it is possible, certainly in the vast majority of cases, if not in all, to readily and easily distinguish between raw and cooked milk or that which has been sterilized at a temperature of 70°C . or higher for short intervals.

(3) The mixture (peroxidase, hydrogen peroxide and phenol¹) had been found to exhibit many analogies to the oxidases. These analogies will be discussed at greater length in a subsequent communication.

(4) That while milks which have been heated to 70°C . for one hour or 75°C . for 20 minutes no longer give the peroxidase

¹ The term "phenol" is here employed in its general sense.

reaction, this reaction is not diminished but, if anything, somewhat intensified by heating the milk to 60° for 20 minutes. It is evident therefore that pasteurization at 60° C. for 20 minutes, as recently recommended by Rosenau, on the basis of his bacteriological studies, does not destroy the biological properties of milk, at least so far as the peroxidase reaction is an indication of such properties.

(5) That the milks of different cows of the same herd exhibit considerable differences in peroxidase activity.

(6) That towards the three peroxidase reagents which we have investigated woman's milk shows decidedly less oxidizing power than cow's milk. For cow's milk, the peroxidase reaction, especially under the sensitizing influence of the phenols, is decisive and constant; for woman's milk, on the other hand, the reaction is in many cases weak and uncertain. As a rule, we have found that human milks during the colostral stage of lactation show greater peroxidase activity than milks exhibiting no colostral properties. This is in harmony with the experience of Gillet.¹

¹ *Journ. de physiol. et de pathol. générale*, iv, pp. 503-518, 1902.

THE INFLUENCE OF ETHER ANAESTHESIA UPON THE EXCRETION OF NITROGEN.

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I. INTRODUCTION.

Many important investigations have been made to determine the influence of chloroform anæsthesia upon the excretion of nitrogen. Vidal,¹ Strassmann,² Drapier,³ Heymans and Debüek,⁴ Salkowski,⁵ Hofmeier,⁶ and Chagnoleau⁷ have contributed some

¹ Vidal: Thèse de Paris, 1897, and *Compt. rend. soc. biol.*, xlviii, 474-476.

² Strassmann: *Virchow's Archiv*, cxv, p. 1, 1889.

³ Drapier: *Influences des anæsthetiques sur la nutrition*, 1886.

⁴ Heymans and Debüek: *Archives de pharmacodynamie*, Gand, 1895.

⁵ Salkowski: *Virchow's Archiv*, cxv, p. 339, 1889.

⁶ Hofmeier: *Berl. klin. Wochenschr.*, xv, p. 230, 1883.

⁷ Chagnoleau: Quoted by Vidal.

of the most important investigations in this field. The researches of Vidal are of especial importance in this connection. This investigator carried out a very elaborate series of experiments on man and lower animals in which he determined among other things, the total nitrogen content of the urine as well as the nitrogen in the form of urea, uric acid, creatinin, hippuric acid and ammonia both before and after placing his subjects under the influence of chloroform for varying lengths of time. He reached the conclusion that all the nitrogenous constituents were increased in amount as a result of the narcosis except hippuric acid, the data indicating that this last constituent was decreased in amount under the influence of the chloroform. Vidal's experiments on man were of two kinds, i. e., after operations and after surgical explorations. Under these conditions the excretion of nitrogen was invariably increased, the maximum output being somewhat irregular and occurring in some instances on the first day after the termination of the anæsthetic state and in other instances being delayed until the third day. The chloroform occasionally increased the nitrogen output to three times the normal value. His experiments on dogs and rabbits verified his findings on man.

Strassmann, from a series of observations on dogs, came to the conclusion that chloroform anæsthesia was able to increase the output of nitrogen about 20 per cent and that this increase was generally maintained about forty-eight hours after which time the excretion sank to the normal level. Salkowski found the nitrogen content of the urine of a dog to be increased after introducing 200 cc. of chloroform water into the animal's stomach, whereas Heymans and Debüek observed similar phenomena to follow the hypodermic administration of chloroform to well-nourished and fasting rabbits. In the case of the fasting rabbits the output of urea was tripled. Drapier found the urea content of the urine of six men to be decreased from one to three grams in the twenty-four hours following chloroform narcosis, whereas in four other cases he could detect practically no variation in the urea excretion after the induction of the anæsthesia from what it had been prior to the administration of the chloroform. His data indicate that the urea output was frequently increased two to four grams in the urine first voided after narcosis, whereas when the whole twenty-four hours was taken into account this

increase was compensated and the ultimate result was a twenty-four hour urea excretion very closely approximating the normal. Hofmeier noted a pronounced increase of urea in the urine of twenty-four women in labor after the administration of from 15 to 100 grams of chloroform. Chagnoleau found no change in the urea excretion after chloroform anæsthesia.

Fewer in number and of less importance are the investigations which have been made to determine the influence of ether anæsthesia upon the output of nitrogen. Schipilin,¹ Kappeler,² Leppmann,³ and Taniguti⁴ have investigated the problem, the observations of Leppmann being of especial interest. This investigator used dogs, cats, guinea pigs and white rats as subjects. Regarding the influence of ether anæsthesia upon the excretion of nitrogen he concluded that this form of narcosis was followed by an increased output of nitrogen but that the increase was not so pronounced as that which followed the administration of chloroform. In one experiment in which a dog in a condition of nitrogen equilibrium was subjected to ether anæsthesia for a period of three hours the nitrogen content of the urine was increased over 60 per cent above the normal on each of the three succeeding days. Keppeler experimented on man and observed a decrease in the excretion of urea to follow ether anæsthesia in thirteen cases out of twenty, whereas five of the remaining seven cases exhibited an increased urea output and in the two remaining instances no change was noted in the urea excretion. Hawk and Gies⁵ noted a decrease in the excretion of nitrogen by a dog subjected to ether-chloroform anæsthesia, whereas when the anæsthesia was accompanied by operation the output of nitrogen was increased. These opposed findings were obtained in experiments made upon different dogs. Taniguti subjected a dog to ether narcosis for a period of three hours and found that the excretion of nitrogen was increased from 14.4 to 14.8 grams per day, an increase of 4.1 per cent. Taniguti subjected this

¹ Schipilin: *Vrachi*, p. 365-368, 1892.

² Kappeler: *Anæsthetica*, Stuttgart, p. 219, 1880.

³ Leppmann: *Mitteilungen aus den Grenzgebieten der Medizin und Chirurgie*, iv, p. 21, 1899.

⁴ Taniguti: *Virchow's Archiv*, cxx, p. 121, 1890.

⁵ Hawk and Gies: *Amer. Journ. of Physiol.*, xi, p. 171, 1904.

same animal to chloroform anæsthesia for a period of two and one-half hours and observed an increase of 5.6 grams or 9.1 per cent in the nitrogen output for the four days following.

The idea of making a thorough study of the various phases of the influence of ether anæsthesia upon metabolism in general as well as upon the hæmoglobin content and the morphological constituents of the blood of well-nourished and of fasting animals following such anæsthesia was suggested in connection with an investigation made by the author in collaboration with Professor Gies at the College of Physicians and Surgeons, New York.¹ The results of this study will be embraced in a series of papers of which the present article is the first. A preliminary report of some of the general points in connection with the influence of ether anæsthesia has already been made.² The purpose of the investigation represented by the experimental data included on the following pages was to determine the influence of ether anæsthesia upon the excretion of total nitrogen, in dogs which were in nitrogen equilibrium.

The length of the periods of anæsthesia ranged from one-half hour to four and one-half hours. Each experiment, as indicated by the data, consisted of three periods, a preliminary period during which the dog was brought into nitrogen equilibrium, an anæsthesia period during which anæsthesia was induced for a definite length of time and its influence noted, and a final period following the termination of the anæsthetic state during which the dog was again brought into a condition of nitrogen equilibrium. Making, as we did, several experiments in series, to show the influence of anæsthesia during periods of different length upon the same animal we found it convenient to use the two or three final days of one experiment as the preliminary period of another experiment. Anæsthesia was induced only when the animal was at nitrogen equilibrium in every instance except in Experiment III, where the same animal was subjected to periods of anæsthesia of one hour, two hours and three hours, respectively, on three consecutive days. This was done to deter-

¹ Hawk and Gies: *Loc. cit.*

² Hawk: Proceedings of the American Physiological Society, *Amer. Journ. of Physiol.*, x, p. xxxvii, 1904.

mine whether there was any appreciable *cumulative* effect of the anæsthesia under these conditions.

Squibb's ether was the anæsthetic employed in every experiment.

II. GENERAL DESCRIPTION.

1. *Subjects, methods, etc.* Four dogs were used as subjects, during the progress of these tests, the body weights of the different subjects ranging from 6.53 to 10.18 kilos. These animals were kept in cages especially adapted for the accurate collection of all excreta¹ and were fed a suitable, accurately weighed, uniform diet (see Table I, p. 343) until nitrogen equilibrium was reached, this diet being fed for several days before any excreta were collected for analysis. As soon as it was certain that the dog was in a condition of nitrogen equilibrium, as judged from analyses of the food and excreta, the animal was subjected to the influence of ether narcosis according to the customary procedure and the nitrogen content of the excreta for a certain length of time following the anæsthesia was determined. The urine during the days preceding the anæsthesia period was collected in twenty-four hour samples, the day ending at 5 p.m. For periods ranging from twenty-four hours to seventy-two hours after the subjection of the animal to the influence of ether narcosis, the urine was collected in *fractions as normally voided* by the beast and each individual fraction analyzed. In connection with experiments upon nitrogen metabolism such as the present study embraces,² the author prefers the method of collecting the urine as normally voided by the animal to the custom of removing the urine by means of a catheter. The collection of each individual fraction of urine voided by the subject during a period of twenty-four hours or more following the anæsthesia and the chemical analysis of those fractions was a rather time-consuming arrangement. However, we considered that the most accurate data regarding the relation existing between the time the animal was placed under the influence of ether and the appearance in the urine of any indications of the influence of such narcosis could only be obtained by carefully examining the specific fractions as voided. We believe the accuracy of our deductions has been greatly increased by the adoption of such a plan of urine collection. In some instances where the urine fractions were of very small volume it was necessary to dilute the specimen with water before subjecting it to analysis, making proper correction for the amount of water used in the dilution.

The experimental day ended at 5 p. m. and therefore all urine passed after 5 p. m. any day will be mentioned in the record as being excreted on the day following. For instance, in Experiment VII the fraction containing 0.54 gram of nitrogen which is recorded as the first fraction voided on the fourth day was passed at 7 p.m. of the third day, and further the

• ¹ Hawk: *University of Pennsylvania Medical Bulletin*, December, 1905.

² Hawk and Gies: *Loc. cit.*

fraction containing 2.68 grams of nitrogen which is recorded as the second fraction of the fourth day was in reality excreted at 11:20 p. m. of the third day.

All nitrogen determinations in foods, feces, urine, etc., were made by a modification of the Kjeldahl method which includes the preliminary digestion of the material by means of sulphuric acid and cupric sulphate.¹

2. *Diet, preparation and preservation of the beef, etc.* The diet of the dogs consisted of beef, cracker dust, lard, bone ash and water, the amounts fed depending, of course, upon the body weight of the animal (see Table I, p. 343). The beef, constituting as it does the main source of the nitrogen of such a diet, is naturally of first importance in experiments of this character. For this reason special care was exercised in the preparation and preservation of the beef fed during the investigation. It was bought in large amount, rendered as free as possible from fat and connective tissue, subsequently hashed by means of an ordinary meat grinder and the juice thoroughly expressed in a tincture press.² After being thoroughly pressed, the various portions were intimately mixed, samples taken for analysis, and the remainder enclosed in sheets of paraffine paper of suitable size, and pressed into the form of cakes about three-fourths of an inch in thickness. These cakes were transferred to large museum jars which were then sealed and placed in the cold-storage room. The beef froze in hard cakes, but the cakes were very easily removed from the jar, since the paraffine paper prevented them from freezing to each other or to the surrounding glass. When needed for use, a cake was removed, the amount of beef desired was accurately weighed and the unused portion returned to the jar in its original paraffine paper covering. Chemical analyses of the beef used in this investigation and prepared and preserved as indicated above, showed the beef to have undergone no appreciable alteration in the content of nitrogen, phosphorus and sulphur during a period of about four months.³

3. *Collection of the excreta and their preparation for analysis; care of the dogs, etc.* As has already been indicated, the urine samples were collected in twenty-four hour periods on all days with the exception of the period immediately following anaesthesia, at which time the fractions were collected as voided. All urine samples were preserved with powdered thymol and as a further precaution they were kept in a cool place until all analytical data had been obtained. The feces was removed from the cage as soon as passed and the fresh weight of the stool was then determined. The fecal mass was now carefully dried below 100° C. and the weight of the dry material determined, after which the feces was carefully powdered and an aliquot portion taken to make up a composite example. The feces for each experimental period, i. e., *preliminary, anaesthesia and final* periods was analyzed separately. The hair, scurf, etc., were removed from the

¹ Hawk: *Practical Physiological Chemistry*, p. 359, 1907.

² Gies: *Amer. Journ. of Physiol.*, v, p. 235, 1901.

³ Hawk: *University of Pennsylvania Medical Bulletin*, December, 1905.

sliding tin bottom of the cage every day at 5 p.m. when the dogs were fed. These were collected in periods corresponding to the feces periods. After cutting the hair into exceedingly short pieces by means of scissors it was intimately mixed with the scurf, etc., and after drying at 110° C., subjected to analysis in the usual way. During the course of an experiment of this sort a certain small amount of urine will dry upon the metal sides and bottom of the cage together with traces of feces particles, scurf and hair, and these can only be removed by careful washing. It was therefore customary to subject the metal portions of the cages to a thorough scrubbing at the end of each experimental period, the water used in the process and its contained urine, feces particles, etc., being made up to a definite volume and subjected to analysis. It was ordinarily found expedient to filter the hair, scurf and feces particles from such washings and add them to the hair and scurf removed from the cage bottom during the course of the period.

III. EXPERIMENTAL DATA.

1. *Discussion of Experiment I.* The subject of this experiment was a brown bitch, weighing 6.53 kg. The preliminary period of the experiment extended over four days during which time the daily excretion of nitrogen ranged from 3.16 grams to 4.26 grams. The nitrogen balance for the period showed an average daily loss of but 0.03 gram (Table XV, p. 351), a condition eminently satisfactory under which to begin the study of the influence of anæsthesia. On the morning of the fifth day the animal was subjected to ether anæsthesia from 9:07 o'clock to 10:07 o'clock, a period of one hour. In the preliminary anæsthesia the beast struggled rather more strenuously than is generally the case. During the semi-unconscious condition following deep anæsthesia the dog was observed to tremble violently, the trembling continuing for more than an hour after the beast regained complete consciousness.

The average daily nitrogen excretion for the four days of the preliminary period was 3.76 grams (see Table II, p. 343). This was increased for the next two days to an average of 4.38 grams, an increase of 0.62 gram or 16.5 per cent per day. During the final period the normal rate of excretion was resumed, the average daily excretion of nitrogen for the four days of the period being 3.85 grams which was only 0.09 gram per day above the average daily excretion for the preliminary period. If we consider the influence of the narcosis as extending over 3 days instead of two

the average daily nitrogen excretion for those days was 4.04 grams as against 4.38 grams as an average for two days, thus lowering the average daily increase from 0.62 gram to 0.28 gram and lowering the average daily percentage increase from 16.5 per cent to 7.4 per cent. Further, if we extend the period in order to include four days we observe that the average daily excretion of nitrogen was 4.14 grams, thus making the average daily increase in the nitrogen excretion 0.38 gram or 10.1 per cent. Taking into consideration the entire experiment from the time anæsthesia was induced until the end of the final period it will be noted that the average daily increase in the nitrogen excretion was 0.27 gram or 7.1 per cent. It is thus seen that, in the case of this subject, the ether narcosis for a period of one hour caused an increased excretion of nitrogen through a period of six days, the minimum increase (16.5 per cent) occurring during the early part of the period, and further that the increase was not temporary, but continued throughout the remainder of the experiment, showing an increase of 7.1 per cent for the entire experimental period following anæsthesia.

2. *Discussion of Experiment II.* The same bitch was used in this experiment as was used in Experiment I, her body weight now being 6.18 kg. During the preliminary period the dog was excreting an average of 3.67 grams of nitrogen per day and the balance for the two days of the period (Table XV, p. 351) shows a daily loss of 0.05 gram of nitrogen. Since the animal was in such excellent condition, as regarded equilibrium, it was not necessary to prolong the preliminary period and therefore on the third day of the experiment the dog was again placed under the influence of ether. On this occasion the period of anæsthesia continued from 9:31 a.m. to 11:31 a.m., a period of two hours or double the length of the anæsthesia period of Experiment I. In this instance the violent trembling, which was so apparent the first time the animal was anæsthetized, was not especially noticeable although the period of anæsthesia was 100 per cent longer.

By referring to Table III, p. 343, it will be observed that the average daily excretion of nitrogen at the time the anæsthetic was administered was 3.67 grams. During the day of anæsthesia and the following day however this average was increased to

4.20 grams, an average daily increase of 0.53 gram or 14.4 per cent. This high plane of the nitrogen excretion was fairly well maintained for the succeeding three days, the data for the five days including the day of anæsthesia showing an aggregate increase of 2.40 grams which was an average daily increase of 0.48 gram or 13.1 per cent over the daily average of the preliminary period. Taking the entire final seven days into consideration it is found that the two hours anæsthesia caused an increase in the amount of nitrogen excreted during the period aggregating 2.59 grams or an average daily increase of 0.37 gram or 10.1 per cent above the normal equilibrium plane for this animal. As contrasted with the effect of the influence of anæsthesia continued for a period of one hour on this same animal as set forth in the first experiment, it is seen that the period of narcosis two hours in duration was instrumental in causing a less pronounced immediate increase in the excretion of nitrogen whereas the ultimate increase brought about in the nitrogen output was greater after two hours anæsthesia. The high level to which the nitrogen excretion was raised through the administration of the anæsthetic was maintained more nearly in this case than in the first experiment. The data showed an average daily increase of 7.1 per cent in the excretion of nitrogen under the influence of anæsthesia for one hour as compared with an average daily increase of 10.1 per cent under the influence of a two-hour anæsthesia period.

3. *Discussion of Experiment III.* The brown bitch which served as subject in Experiments I and II was again employed. Her body weight was now reduced to 5.96 kg. The plan of this experiment differed materially from the plans of Experiments I and II, already discussed. In the first two experiments as we have already seen, there was but a single anæsthesia period and the anæsthetic was administered when the dog was in a condition of nitrogen equilibrium. In this third experiment however there were three periods of narcosis, i. e., the animal was placed under the influence of ether at three different times on as many consecutive days, no attempt being made to secure nitrogen equilibrium between the various anæsthesia periods. The plan was to get the animal into nitrogen equilibrium, then induce anæsthesia for a period of one hour, this to be followed after twenty-four hours by an anæsthesia period of two hours' duration and this in

turn at the end of another twenty-four hours time to be followed by a third application of the anæsthetic through a period of three hours. From a consideration of the data for experiments in which the dog was subjected to but a single anæsthetization it is evident that the full effect upon the excretion of nitrogen of the anæsthesia thus produced, can not be measured by an examination of the urine voided during the day upon which the anæsthetic was administered. Therefore, it is obvious, that, in subjecting the same animal to ether narcosis for periods of varying lengths on three consecutive days, we were producing anæsthesia at the second and third narcoses in an organism which had not yet fully recovered from the influence of the previous administration of the anæsthetic. By this procedure we hoped to be able to secure data upon the cumulative effect of ether anæsthesia under these conditions. At the time the experiment began the dog was excreting an average of 3.77 grams of nitrogen per day (Table IV, p. 344) and the nitrogen balance for the preliminary period (Table XV, p. 351) showed a daily loss of 0.10 gram. Such was the condition of the dog when she was placed under the influence of ether. Anæsthesia began at 9:32 a.m. on the third day of the experiment and continued for one hour or until 10:32 a.m., the beast being etherized with less difficulty than in either of the preceding experiments. The day following she was etherized from 10:04 a.m. to 12:04 p.m. and on the third day she was under the influence of the anæsthetic from 9:05 a.m. until 12:05 p.m. Following this last narcosis the beast slept practically the remainder of the day.

As has already been observed, the average daily output of nitrogen in the urine of the preliminary period was 3.77 grams. The influence of the one hour period of anæsthesia was evidenced by an increase of 1.03 gram of nitrogen during the day the ether was administered. This represents a 27.1 per cent increase. Upon the following day however, notwithstanding that the animal was under the influence of ether narcosis for a period of two hours, the excretion of nitrogen for the day was normal (3.75 grams). The influence of the anæsthesia was apparently somewhat belated in this case, for upon the next day when the animal was anæsthetized for a period of three hours the analysis of the urine showed the surprisingly large output of 5.41 grams of

nitrogen, an increase for the day of 1.64 gram above the normal output. This increased excretion of 1.64 gram or 43.5 per cent upon the third day of anæsthesia was by far the *greatest increase in nitrogen secured in any of our experiments upon the influence of anæsthesia* and illustrates very strikingly the cumulative effect of the narcosis. The first general effect of the anæsthesia may be assumed to have ended upon the sixth day of the experiment. Taking into consideration days 3 to 6 we find that the ultimate influence of the anæsthesia induced for periods of one, two and three hours respectively upon three consecutive days was to cause an average daily excretion of nitrogen amounting to 4.75 grams. This represents an average daily increase of 0.98 gram or 25.9 per cent for a period of four days, and was the maximum effect of ether narcosis through a period of days as shown by our experiments. It will be noted, from an examination of Table IV, p. 344, that the period of maximum nitrogen excretion, i. e., days 3 to 6, was followed by a period of four days during which the excretion of nitrogen was on a much lower level. From this point on to the end of the experiment however the excretion of nitrogen was on a somewhat higher plane, although considerably below the level of the period of maximum excretion (days 3 to 6).

The ultimate influence of the three anæsthesia periods, as measured by the nitrogen output for the entire experiment from the time of the first administration of the anæsthetic, is represented by an absolute increase of 9.30 grams of nitrogen. The average daily increase in the nitrogen excretion for this period was 0.66 gram or 17.5 per cent.

4. *Discussion of Experiment IV.* The subject of this experiment was a spotted bitch, weighing 7.72 kg. Fed for several days on a diet containing 5.032 grams of nitrogen daily (see Table I, p. 343), her body was finally brought into a condition of nitrogen equilibrium. At the time the ether was administered she was excreting an average of 4.65 grams of nitrogen per day, her daily balance for the preliminary period being -0.07 gram (see Table XV, p. 351). Upon the fourth day of the experiment proper the beast was subjected to ether narcosis for a period of one-half hour, i. e., from 8:15 a.m. to 8:45 a.m. This animal showed a particularly rapid recovery from the effect of the anæsthetic, and at 9:10 a.m., or twenty-five minutes after the termination of the anæ-

thetic state, she was as bright and active as usual and seemingly entirely free from any influence of the ether. This was in marked contrast to the recovery noted in the case of the subject of the first experiment.

In the case of this subject, the initial influence of the anæsthetic upon the excretion of nitrogen seemed to persist with comparatively little variation for a period of 48 hours, as will be seen by an examination of Table V, p. 345. The average daily excretion of nitrogen for the preliminary period was 4.65 grams. Upon the day of anæsthesia the output of nitrogen was increased to 4.97 grams and this output was practically duplicated (4.94 grams) upon the next day. Therefore the effect of ether narcosis for a period of one-half hour was to increase the excretion of nitrogen 0.61 gram for a period of two days, this being an average daily increase of 6.5 per cent. An examination of Table V, p. 345, will show that a decided decrease in the output of nitrogen occurred on the sixth day of the experiment, placing the total excretion for the day far below normal and that this subnormal elimination was followed by a day of high nitrogen excretion. The nitrogen output for the final day of the experiment was again below normal. Taking into consideration the days 6 to 8 which followed immediately after the days during which the influence of the anæsthesia was most strikingly shown, we observe that the average daily nitrogen excretion for this period of three days was 4.33 grams. This was 6.9 per cent below the normal output. Calculating the nitrogen excretion for the entire experiment after anæsthesia we observe that the average daily output was only 4.58 grams which was 0.07 gram or 1.5 per cent below the normal nitrogen level as maintained during the preliminary period. Therefore, notwithstanding the fact that during the first two days after anæsthesia the excretion of nitrogen was considerably increased, sufficiently increased in fact to amount to an average daily increase of 6.5 per cent for the period, yet the aggregate excretion for the whole experiment indicates that this initial increase was more than counterbalanced during the following days. Therefore instead of causing a permanent increase in the excretion of nitrogen, the ultimate influence of the one-half hour of ether anæsthesia was to cause an average daily *decrease* of 0.07 gram or 1.5 per cent in the output of nitrogen. In each of our other

experiments up to this time, the general effect of the ether narcosis was to cause a more or less marked *increase* in the excretion of nitrogen. The conditions surrounding this experiment differed from the conditions of those experiments which preceded it only in that the period of anæsthesia was shorter (one-half hour instead of one to three hours) and that the subject recovered more rapidly from the effect of the narcosis.

5. *Discussion of Experiment V.* For this experiment a dog weighing 10.18 kg. was used as subject. He was fed a ration containing 7.17 grams of nitrogen per day for several days and at the time the anæsthetic was administered the animal showed a plus balance of 0.09 gram of nitrogen per day (see Table XV, p. 352). This was the first instance of a plus balance during a preliminary period up to this point in the experiments. Upon the fourth day of the experiment anæsthesia was induced at 9:15 a.m. and continued for one hour or until 10:15 a.m. The dog struggled very violently and salivated profusely during the preliminary anæsthesia. Ten minutes after being returned to his cage he began making vain attempts to stand and continued falling about the cage until 11:15 a.m. when he was partially successful in his efforts to maintain his equilibrium.

During the preliminary period of three days the dog exhibited an average daily excretion of nitrogen amounting to 6.55 grams. Under the influence of the anæsthetic this average excretion was increased to 7.11 grams upon the day of anæsthesia. This represented an increase of 0.56 gram or 8.5 per cent for the day and was the first instance in which the initial influence of the narcosis was confined to the day upon which the anæsthetic was administered. In this experiment it will be noted that each of the days following the day of anæsthesia witnessed a subnormal elimination of nitrogen (see Table VI, p. 345). Computing the influence of the anæsthesia for the whole of the period following the administration of the anæsthetic, we observe that the one hour of anæsthesia caused a *decrease* of 1.58 gram in the excretion of nitrogen for the six days. This was equivalent to an average daily *decrease* of 0.26 gram or 4.0 per cent for the period. The conditions obtaining in this experiment, as regards the influence of the anæsthesia upon the excretion of nitrogen were similar to those observed in Experiment IV, i. e., an initial increase in the excretion of

nitrogen followed by a more pronounced and prolonged decrease of sufficient magnitude to more than counterbalance the influence of the initial increase and to cause the ultimate effect of the anaesthesia to be a *decrease* in the excretion of nitrogen. The data from Experiments IV and V are very similar on this point and differ mainly from the fact that in the former experiment the initial increased excretion of nitrogen extended over a period of two days, whereas in Experiment V this initial increase was confined to the day of the narcosis.

6. *Discussion of Experiment VI.* The subject of this experiment was the animal used in Experiment V, the body weight being 9.70 kg. at the time anaesthesia was induced. At this time the nitrogen balance (Table XV, p. 352) for the beast showed a gain of 0.18 gram per day. This was the poorest balance secured by us in any preliminary period during the series of experiments. At 9:05 a.m. upon the third day of the experiment the dog was fully under the influence of ether and the narcosis was continued until 11:05 a.m. or for a period of two hours. Nothing unusual occurred during the administration of the anaesthetic or the subsequent recovery from its influence. The beast tumbled about the cage somewhat violently during the recovery, and a period of about 20 minutes elapsed before he was able to stand.

The data in Table VII, p. 345, indicate that the average daily excretion of nitrogen during the preliminary period was 6.47 grams. Under the influence of the two hours of anaesthesia the excretion rose to 7.32 grams during the same day. This was an increase of 0.85 gram or 13.1 per cent for the day. The average daily excretion for the remainder of the experiment was also somewhat above the normal. Computing the influence of the anaesthesia for the entire period after anaesthetization we see that the effect of anaesthesia for two hours was to cause a total increase of 1.15 gram in the excretion of nitrogen. This makes the average daily excretion of nitrogen for this period 6.70 grams as compared with an average of 6.47 grams for the preliminary period and represents an average daily increase of 0.23 gram or 3.5 per cent for that portion of the experiment following the administration of the ether.

7. *Discussion of Experiment VII.* The subject of this experiment was the dog used in Experiment VI, the animal weighing

9.51 kg. at the time he was subjected to the influence of ether narcosis. The nitrogen balance for the preliminary period of this experiment shows an average daily loss of 0.04 gram (see Table XV, p. 352). The animal was placed under the influence of ether on the morning of the third day of the experiment for a period of four and one-half hours, the anæsthesia beginning at 8:58 a.m. and ending at 1:28 p.m. The beast ceased breathing after having been under the influence of ether about five minutes, but artificial respiration was successfully employed and the subsequent anæsthesia was continued satisfactorily. The dog appeared stuporous and inactive during the entire day and was apparently asleep a portion of the time. However he brightened up at the smell of food at meal time and ate as usual.

The average daily nitrogen excretion during the preliminary period (Table VIII) was 6.64 grams. The narcosis failed to increase the excretion of nitrogen on the day the anæsthetic was administered but the excretion continued at practically the same level for the day of anæsthesia as it had maintained during the preliminary period. This phenomenon is all the more noteworthy when one realizes that the urine volume for this day was increased 24.8 per cent, a greater increase than was noted in any other instance during the course of the experiments. It is evident that the initial influence of the anæsthesia was simply delayed somewhat for upon the day after the ether was administered the excretion of nitrogen was raised to 7.69 grams, an increase of 1.05 gram or 15.8 per cent. From this point the excretion was subnormal continuously until the close of the experiment with the exception of the eighth day, at which time the excretion was 0.22 gram above normal. Basing our calculations upon the nitrogen excretion for the whole experimental time following the narcosis period of four and one-half hours we observe that the ultimate effect of the ether was to cause an actual total *decrease* of 0.83 gram in the excretion of nitrogen for the seven days. Therefore, the four and one-half hours of anæsthesia was instrumental in producing an average daily decrease of 0.12 gram or 1.8 per cent in the excretion of nitrogen for a period of seven days, notwithstanding the fact that the nitrogen output for the day following the administration of the anæsthetic exhibited an increase aggregating 1.05 gram or 15.8 per cent.

8 *Discussion of Experiment VIII.* The dog used in this experiment was the same animal which served as subject in Experiments V, VI and VII. At the time anaesthesia was induced his weight was 9.23 kg. Maintained upon the same diet as that previously employed the animal showed an average daily nitrogen balance of + 0.16 gram for the preliminary period (see Table XV, p. 352). With the body in this condition the dog was subjected to ether narcosis for a period of one hour upon the fourth day of the experiment. Anaesthesia began at 9:55 a.m. and ended at 10:55 a.m. The animal was somewhat stuporous throughout the day upon which the anaesthetic was administered and slept soundly a portion of the afternoon.

The excretion of nitrogen during the preliminary period averaged 6.47 grams (Table IX, p. 346), this being the same average output observed in the preliminary period of Experiment VI made upon this same subject. During the day of anaesthesia 7.08 grams of nitrogen was excreted, an increase for the day of 0.61 gram or 9.4 per cent above normal. The nitrogen excretion for the next three days was subnormal, the average daily decrease below the daily output of the preliminary period being 0.49 gram or 7.5 per cent. From the eighth day to the end of the experiment (ten days) there was a well marked tendency for the excretion of nitrogen to run on an unusually high plane. This increase in the excretion of nitrogen cannot be explained through the diuretic influence of the anaesthetic since the urine volume for this period was only 6.7 per cent above normal. Taking three consecutive days of this period (13 to 15) into consideration we find that the average daily output of nitrogen for those days was 7.74 grams. This daily average represents an increase of 1.27 gram or 19.6 per cent above normal. Considering the whole of the experiment from the time anaesthesia was induced until the end of the final period, we find that the average daily excretion of nitrogen was 7.12 grams. This shows an average daily increase in the nitrogen excretion amounting to 0.65 gram or 10 per cent for the fourteen days.

9. *Discussion of Experiment IX.* The subject of this experiment was a spotted bitch weighing 7 kilos at the time she was subjected to ether anaesthesia. This dog was a most difficult animal to bring into a condition of nitrogen equilibrium. Over two

weeks were consumed in the preliminary feeding, the ration being changed twice before she was finally brought into a suitable condition for metabolism work. During the six days preliminary to the anæsthesia the average daily nitrogen balance, as shown by Table XV, p. 352, was + 0.10 gram. Ether narcosis was induced from 12:15 p.m. until 1:15 p.m. on the seventh day of the experiment. The dog again proved her individuality by being the most difficult of any of the dogs experimented upon, to anæsthetize. She also salivated far more copiously than did the subjects of the former experiments.

The average daily output of nitrogen for the preliminary period was 5.70 grams (see Table X, p. 346). Under the influence of the one hour period of anæsthesia the excretion of nitrogen was increased to 6.49 grams on the day the animal was placed under the influence of ether. This was an increase of 0.79 gram or 13.9 per cent. The high level of the nitrogen excretion was maintained fairly uniformly for a period of six days and from this point a gradual decrease began, sufficient in extent to bring the output of nitrogen at the end of the sixteenth day of the experiment, practically to the normal level as observed during the preliminary period. The average daily output of nitrogen for the three days following anæsthesia was somewhat higher than the average for any other period during the experiment and computing upon this basis we find that the effect of the ether narcosis induced for one hour was to cause an increase in the excretion of nitrogen, over a period of three days, amounting to 1.9 gram. The average daily increase was 0.63 gram or 11.1 per cent. Computing the influence the anæsthesia exerted upon the excretion of nitrogen throughout the entire portion of the experiment after anæsthesia we observe that the total increase aggregated 3.38 grams, which was an average daily increase of 0.34 gram or 5.9 per cent.

IV. THE SPECIFIC INFLUENCE OF ETHER NARCOSIS UPON THE EXCRETION OF NITROGEN.

In Experiment I the influence of one hour of anæsthesia upon Dog No. 1 was studied. It will be observed by an examination of the data in Table XI, p. 347, that the initial effect of this nar-

cosis upon the excretion of nitrogen was to cause an average daily increase of 0.62 gram or 16.5 per cent for a period of two days after the anæsthetic was administered. From this point until the close of the experiment the average daily excretion was only slightly above normal, the ultimate average daily increase for the entire experiment from the time anæsthesia was induced being 0.27 gram or 7.1 per cent. The total increase in the excretion of nitrogen for a period of six days was 1.62 gram. Following this in Experiment II. notwithstanding the fact that the length of anæsthesia was double that in Experiment I, we observe that the initial average daily increase in the output of nitrogen was 0.53 gram, or 14.4 per cent, an initial daily average somewhat lower than that observed in Experiment I. The excretion of nitrogen during the final period of Experiment II however was on a somewhat higher plane than was noted in the final period of Experiment I, the ultimate average daily increase being 0.37 gram or 10.1 per cent.

The data from Experiment III, in which the animal was subjected to three periods of anæsthesia with no opportunity to return to nitrogen equilibrium between the anæsthesia periods, is of course not strictly comparable with the data from Experiments I and II, although the same dog was used as subject (see Table XI, p. 347). The data from the influence of the first administration of the anæsthetic in Experiment III are however justly comparable with the result of Experiment I in which the anæsthesia was of the same duration, i. e., one hour. By making this comparison we observe that the one hour of anæsthesia in Experiment III caused an increase of 1.03 gram or 27.1 per cent in the excretion of nitrogen for the day of anæsthesia as compared with an initial average daily increase of 0.62 gram or 16.5 per cent in Experiment I. On the second day of Experiment III when the dog was placed under the influence of ether for two hours the output of nitrogen was approximately normal whereas on the third day when the beast was subjected to the influence of the anæsthetic for a period of three hours the output of *nitrogen for the day was increased 1.64 gram or 43.5 per cent*, an increase which represents the *maximum effect of ether anæsthesia* which was observed during the investigation. Taking into consideration the three days of the experiment upon which narcosis was

induced we find that the average daily increase in the nitrogen excretion was 0.47 gram or 11.2 per cent. The level of the nitrogen excretion continued high for the day following also, and taking this day into consideration we observe that the average daily increase in the nitrogen output for the four days including the 3 days of anæsthesia and the day following was 0.57 gram or 13.6 per cent. The cumulative effect of the anæsthesia upon the excretion of nitrogen was portrayed quite decidedly in this experiment inasmuch as the ultimate average daily increase for the entire period of fourteen days following anæsthesia was 0.66 gram or 17.5 per cent, this being the maximum ultimate increase in the nitrogen output observed during our study of ether anæsthesia. In the course of the fourteen days the absolute increase above normal in the excretion of nitrogen aggregated 9.30 grams.

In Experiment IV ether narcosis for one-half hour produced an initial average daily increase of 0.31 gram or 6.5 per cent in the nitrogen output for a period of two days after anæsthesia although the ultimate effect of the anæsthetic, if calculated upon the basis of the whole experimental time following anæsthesia, was to cause an average daily *decrease* of 0.07 gram or 1.5 per cent in the output of that element. The initial average daily increase in the excretion of nitrogen following the administration of the anæsthetic for a period of one-half hour was the *minimum initial increase* observed during the course of our experiments, a result which is perfectly comparable with the length of the anæsthesia period.

Experiment V was the first of a series of four experiments made upon Dog No. 3. The length of the anæsthesia period was one hour. The initial increase in the excretion of nitrogen was 0.56 gram or 8.5 per cent for the day of anæsthesia (Table XI, p. 347) and constitutes the *minimum increase* in the excretion of nitrogen as observed after a *one-hour anæsthesia period*. In this respect the course of the urine volume and its nitrogen content ran parallel since the initial increase in the urine output for this experiment was the *minimum* for a one-hour period of anæsthesia. The ultimate effect of the anæsthesia is represented by an average daily *decrease* of 0.26 gram or 4 per cent in the excretion of nitrogen which was the *maximum average decrease* observed during the

course of the series of nine experiments. The excretion of nitrogen was decreased from the normal a total of 1.58 gram during the six days following narcosis. In Experiment VI the initial average daily excretion of nitrogen, was considerably higher after the two hours of anæsthesia (0.85 gram or 13.1 per cent) than after the one hour period of anæsthesia (0.56 gram or 8.5 per cent) in Experiment V, and further the ultimate effect of the one hour of anæsthesia was an average daily *decrease* in nitrogen whereas the ultimate effect of the two-hour period of narcosis was an average daily *increase* in the nitrogen output. We further observe that the *decrease* after one hour of anæsthesia was greater than the *increase* after the period of anæsthesia extending over two hours. In Experiment VII it is seen that the initial increase in the excretion of nitrogen after four and one-half hours of anæsthesia as observed on the day of anæsthesia was 0.52 gram or 7.8 per cent, an average *daily* increase far below that determined for the excretion of nitrogen in Experiment VI after two hours anæsthesia (0.85 gram or 13.1 per cent) and even lower than the initial increase after a one-hour period of narcosis (0.56 gram or 8.5 per cent) as noted in Experiment V. For the third time during the investigation, the ultimate influence of the anæsthetic was to cause a decrease in the excretion of nitrogen, the ultimate average daily decrease in this instance being 0.12 gram or 1.8 per cent which represents an absolute decrease of 0.83 gram in the excretion of nitrogen for the period of seven days. In Experiment VIII the final test with Dog No. 3 was made, the length of anæsthesia being one hour. An examination of the data will show that the initial average daily increase in the excretion of nitrogen after the one hour of anæsthesia (0.61 gram or 9.4 per cent) was an increase which was very similar to that observed in Experiment V after anæsthesia for a like period (0.56 gram or 8.5 per cent). The ultimate effect of the narcosis in the two cases mentioned however was decidedly different, inasmuch as the one hour of anæsthesia in Experiment V caused an ultimate average daily *decrease* of 0.26 gram or 4 per cent in the nitrogen output, whereas the one hour of anæsthesia in Experiment VIII caused an ultimate increase in the output of nitrogen aggregating 0.65 gram or 10 per cent for the entire final period of fourteen days, this being the *maximum ultimate average*

daily increase for the animal. As a result of this increase, an excess of 7.12 grams of nitrogen above normal was excreted during the period.

In Experiment IX the fourth subject of the investigation, Dog No. 5, was subjected to anæsthesia for one hour. The initial result was an increase of 0.79 gram or 13.9 per cent in the excretion of nitrogen for the day of anæsthesia which was a more pronounced increase than was occasioned in either Experiments I, V or VIII in which narcosis for a similar period was induced on other subjects. The ultimate average daily increase in the nitrogen output amounted to 0.34 gram or 5.9 per cent for the final period of ten days and represents an absolute increase, above normal, in the excretion of nitrogen aggregating 3.38 grams.

V. CONCLUSIONS.

(1) Ether anæsthesia induced in dogs which were at nitrogen equilibrium invariably caused an increase in the excretion of nitrogen during the twenty-four to forty-eight hours following the narcosis.

(2) The minimum initial average daily increase (0.31 gram or 6.5 per cent) in the excretion of nitrogen was secured after a 30-minute period of anæsthesia which was the minimum anæsthesia period of the investigation, however the percentage increase of nitrogen in the various experiments was in general but slightly dependent upon the length of the period of narcosis.

(3) The maximum initial average daily increase in the excretion of nitrogen was 1.64 gram or 43.5 per cent which was observed after 3 hours of anæsthesia, the animal having been subjected to one hour of anæsthesia two days previously and to two hours of anæsthesia on the day immediately preceding that upon which the maximum increase was obtained.

(4) That the influence of ether narcosis upon the excretion of nitrogen was dependent upon the individuality of the subject anæsthetized was indicated by the fact that anæsthesia induced in each of three subjects for a period of one hour was instrumental in increasing the initial average daily percentage output of nitrogen from 8.5 per cent to 27.1 per cent, according to the subject.

(5) The ultimate influence of ether anæsthesia in six of the nine experiments reported was an increase in the output of nitrogen the ultimate average daily increase ranging from 3.5 per cent to 17.5 per cent. In the three experiments which demonstrated a decrease in the excretion of nitrogen the ultimate average daily *decrease* varied from 1.5 per cent to 4 per cent.

(6) That ether, in some cases, has a cumulative influence upon the excretion of nitrogen was shown by the fact that the *first* narcosis of one of our subjects caused an ultimate average daily percentage *decrease* of 4 per cent, whereas the *fourth* narcosis of this subject for a period of equal length was productive of an ultimate average daily percentage *increase* of 10 per cent.

(7) Ether narcosis had no influence upon the feces in any of our experiments, the amount and composition of the fecal matter passed by the various subjects being strikingly uniform throughout the series of tests.

(8) The data from our experiments would seem to indicate that, in all metabolism experiments upon dogs where the animal is subjected to ether anæsthesia, it is necessary to make check experiments to determine as accurately as possible the influence of such anæsthesia and to make the proper correction. At the same time our results further indicate that the possibility of determining what the exact influence of the narcosis was during the experiment proper, by a check test either before or after such experiment, is rather remote, inasmuch as the reaction of the animal to the anæsthetic will vary at the second administration of the anæsthetic from that observed as the result of the first narcosis.

TABLE I. DAILY DIET.

SUBJECT.	BEEF.			CRACKER DUST.			LARD.			BONE ASH.			WATER.
	Grams per day.	Nitrogen.		Grams per day.	Nitrogen.		Grams per day.	Nitrogen.		Grams per day.	Nitrogen.		Grams per day.
		Per cent.	Grams per day.		Per cent.	Grams per day.		Per cent.	Grams per day.		Per cent.	Grams per day.	
Dog No. 1	105	3.52	3.696	20	2.01	0.402	10	0.022	0.002	4	0.020	0.001	250
Dog No. 2	120	3.52	4.224	40	2.01	0.804	15	0.022	0.003	5	0.020	0.001	300
Dog No. 3	175	3.52	6.160	50	2.01	1.005	18	0.022	0.004	6	0.020	0.001	400
Dog No. 5	155	3.52	5.456	40	2.01	0.804	18	0.022	0.004	6	0.020	0.001	350

TABLE II. EXPERIMENT I.

Dog. No. 1.

Day	1	2	3	4	Ether anaesthesia 1 hour.	5	6	7	8	9	10
Nitrogen in urine, { grams.....	3.66	3.16	3.97	4.26		4.21	1.09* 3.45	3.57	4.43	3.76	3.58
Total grams.....	3.66	3.16	3.97	4.26		4.21	4.54	3.57	4.43	3.76	3.58

* First urine after anaesthesia, passed at 7 p.m.

TABLE III. EXPERIMENT II.

Dog. No. 1.

Day	1	2	Ether anaesthesia hours.	3	4	5	6	7	8	9
Nitrogen in urine, { grams.....	3.76	3.58		3.67	1.17* 2.20 1.36	4.03	3.94	4.36	3.36	4.18
Total grams.....	3.76	3.58		3.67	4.73	4.03	3.94	4.36	3.36	4.18

* First urine after anaesthesia, passed at 6:30 p.m.

TABLE IV. EXPERIMENT III.
Dog. No. 1.

Day	1	2	Ether anaesthesia, 1 hour.		3	Ether anaesthesia, 3 hours.		4	Ether anaesthesia, 3 hours.		5	6	7	8	9	10	11	12	13	14	15	16															
Nitrogen in urine, grams.	}	3.36	4.18	4.03		0.77*	0.34		0.74†	5.05	4.11	5.05	4.15	4.20	3.58	4.08	4.52	4.42	4.78	4.19	4.63	4.52															
				0.77*			0.44																														
				0.77*			1.93																														
				0.77*			0.29																														
Total grams.....	}	3.36	4.18	4.80		0.75†	3.75		5.41	5.05	4.15	4.20	3.58	4.08	4.52	4.42	4.78	4.19	4.63	4.52																	
				4.80			3.75																														

* First urine after 1 hour anaesthesia.

† First urine after 2 hours anaesthesia.

‡ First urine after 3 hours anaesthesia.

TABLE V. EXPERIMENT IV.
Dog. No. 2.

Day	1	2	3	Ether anaesthesia 1 hour.	4	5	6	7	8
Nitrogen in urine, grams .. {	4.49	4.50	4.95		3.87 1.10*	4.05 0.89	0.49 3.34	5.02	4.14
Total grams	4.49	4.50	4.95		4.97	4.94	3.83	5.02	4.14

* First urine after anaesthesia, passed at 4:30 p.m.

TABLE VI. EXPERIMENT V.
Dog. No. 3.

Day	1	2	3	Ether anaesthesia 1 hour.	4	5	6	7	8	9
Nitrogen in urine, grams {	6.50	6.29	6.87		5.93 0.26* 0.92	5.20 0.90	1.23 4.30	6.04	6.52	6.42
Total grams	6.50	6.29	6.87		7.11	6.10	5.53	6.04	6.52	6.42

* First urine after anaesthesia, passed at 11:30 a.m.

TABLE VII. EXPERIMENT VI.
Dog. No. 3.

Day	1	2	Ether anaesthesia 2 hours.	3	4	5	6	7
Nitrogen in urine, grams..... {	6.52	6.42		6.06 0.04* 0.22† 0.47 0.53	6.65	6.24	6.45	6.84
Total grams	6.52	6.42		7.32	6.65	6.24	6.45	6.84

* First urine after anaesthesia, passed at 11:20 a.m., 15 minutes after end of anaesthesia.

† Passed at 12:30 p.m.

TABLE VIII. EXPERIMENT VII.
Dog. No. 3.

Day	1	2	Ether anaesthesia 1 1/4 hours.	3	4	5	6	7	8	9
Nitrogen in urine, grams..... {	6.45	6.84		4.82 0.40* 0.96† 0.23 0.22	0.54 2.68 2.72 0.90 0.85	6.37	5.54	6.02	6.86	6.54
Total grams	6.45	6.84		6.63	7.69	6.37	5.54	6.02	6.86	6.54

* Passed during preliminary anaesthesia.

† First urine after anaesthesia, passed at 2:15 p.m.

TABLE IX. EXPERIMENT VIII.

Dog. No. 3.

Day	1	2	3	Ether anaesthesia, 1 hour.		4	5	6	7	8	9	10	11	12	13	14	15	16	17
Nitrogen in urine, grams {	6.02	6.86	6.54	5.72		0.61*	5.77	5.87	5.68	7.45	5.92	8.63	7.63	6.94	8.24	7.26	7.71	7.66	7.20
Total grams.....	6.02	6.86	6.54	7.08			6.38	5.87	5.68	7.45	5.92	8.63	7.63	6.94	8.24	7.26	7.71	7.66	7.20

* First urine after anaesthesia, passed at 12:45 p. m.

TABLE X. EXPERIMENT IX.

Dog. No. 5.

Day	1	2	3	4	5	6	Ether anaesthesia, 1 hour.		7	8	9	10	11	12	13	14	15	16
Nitrogen in urine, grams..... {	5.86	5.81	5.50	5.39	5.97	5.69	5.43		5.43	0.33								
Total grams.....	5.86	5.81	5.50	5.39	5.97	5.69	1.06*		1.06*	0.88								

* First urine after anaesthesia, passed at 4:50 p. m.

TABLE XI.

Influence of Ether Anæsthesia upon the Excretion of Nitrogen.

Experiment No.	Length of anæsthesia.		Average daily excretion of nitrogen during the preliminary period.	AVERAGE DAILY GAIN OR LOSS IN NITROGEN EXCRETION DUE TO THE INFLUENCE OF ANAESTHESIA.				Absolute gain or loss in nitrogen excretion for the entire experiment following anæsthesia.
	hrs.	days.		grams.	grams.	per cent.	grams.	
I	1	6	3.76	+0.62	+16.5	+0.27	+7.1	+1.62
II	2	7	3.67	2 days		6 days		+2.59
				+0.53	+14.4	+0.37	+10.1	
III	6	14	3.77	2 days		7 days		+9.30
				+1.03‡	+27.1‡	+0.66	+17.5	
				-0.02§	-0.5§			
IV	½	5	4.65	+1.64 +43.5		14 days		-0.35
				3 days		5 days		
V	1	6	6.55	+0.31 +6.5		-0.07 -1.5		-1.58
				2 days		6 days		
VI	2	5	6.47	+0.56 +8.5		-0.26 -4.0		+1.15
				1 day		5 days		
VII	4½	7	6.64	+0.85 +13.1		+0.23 +3.5		-0.83
				1 day		7 days		
VIII	1	14	6.47	+0.52 +7.8		-0.12 -1.8		+7.12
				2 days		14 days		
IX	1	10	5.70	+0.61 +9.4		+0.65 +10.0		+3.38
				1 day		10 days		

* Average daily gain or loss noted during 24 to 48 hours after anæsthesia.

† Average daily gain or loss for the entire period from the time of anæsthesia until the experiment closed.

‡ After 1 hour anæsthesia.

§ After 2 hours anæsthesia.

|| After 3 hours anæsthesia.

TABLE XII.
Weight and Nitrogen Content of Feces.

DOG.	Experiment.	PERIOD.	No. of days.	DRY FECEs.	
				Weight.	Nitrogen.
1	I	Preliminary.....	4	<i>grams.</i> 35.6	<i>grams.</i> 1.01
		Anæsthesia.....	2	20.1	0.64
		Final.....	4	39.9	1.31
	II	Preliminary.....	2	19.7	0.66
		Anæsthesia.....	2	21.3	0.71
		Final.....	5	43.9	1.50
	III	Preliminary.....	2	18.2	0.60
		Anæsthesia.....	4	36.1	1.22
		Final.....	10	90.3	3.49
2	IV	Preliminary.....	3	27.0	0.88
		Anæsthesia.....	2	20.1	0.56
		Final.....	3	31.2	0.83
3	V	Preliminary.....	3	36.4	1.11
		Anæsthesia.....	1	13.1	0.34
		Final.....	5	63.1	1.70
	VI	Preliminary.....	2	25.2	0.68
		Anæsthesia.....	1	12.9	0.37
		Final.....	4	51.3	1.48
	VII	Preliminary.....	2	27.2	0.74
		Anæsthesia.....	2	25.3	0.67
		Final.....	5	64.1	1.76
	VIII	Preliminary.....	3	35.3	1.05
		Anæsthesia.....	1	11.0	0.33
		Final.....	13	146.5	3.77
5	IX	Preliminary.....	6	63.2	1.74
		Anæsthesia.....	3	31.5	0.81
		Final.....	7	74.1	1.96

TABLE XIII.

*Weight and Nitrogen Content of Hair.**

DOG.	Experiment.	PERIOD.	No. of days.	HAIR.	
				Weight.	Nitrogen.
1	I	Preliminary.....	4	<i>grams.</i> 4.06	<i>grams.</i> 0.32
		Anæsthesia.....	2	1.98	0.14
		Final.....	4	4.01	0.28
	II	Preliminary.....	2	1.87	0.14
		Anæsthesia.....	2	1.96	0.16
		Final.....	5	4.92	0.30
	III	Preliminary.....	2	1.83	0.12
		Anæsthesia.....	4	4.07	0.28
		Final.....	10	9.23	0.80
2	IV	Preliminary.....	3	3.07	0.27
		Anæsthesia.....	2	1.89	0.18
		Final.....	3	2.97	0.27
3	V	Preliminary.....	3	3.00	0.24
		Anæsthesia.....	1	0.98	0.09
		Final.....	5	4.81	0.45
	VI	Preliminary.....	2	1.91	0.18
		Anæsthesia.....	1	0.93	0.09
		Final.....	4	3.86	0.36
	VII	Preliminary.....	2	1.92	0.18
		Anæsthesia.....	2	1.83	0.20
		Final.....	5	4.71	0.50
	VIII	Preliminary.....	3	2.78	0.30
		Anæsthesia.....	1	0.89	0.09
		Final.....	13	10.62	1.17
5	IX	Preliminary.....	6	5.64	0.54
		Anæsthesia.....	3	2.90	0.24
		Final.....	7	6.67	0.56

* This includes the hair, scurf, etc., brushed from the pan of the cage at the end of each day, as well as that removed from the cage washings by filtration.

TABLE XIV.
Volume and Nitrogen Content of Cage Washings.

DOG.	Experiment.	PERIOD.	No. of days.	CAGE WASHINGS.	
				Volume.	Nitrogen.
1	I	Preliminary.....	4	cc. 3400	grams. 0.24
		Anæsthesia.....	2	2200	0.14
		Final.....	4	3200	0.32
	II	Preliminary.....	2	2500	0.16
		Anæsthesia.....	2	2400	0.14
		Final.....	5	3200	0.35
	III	Preliminary.....	2	2100	0.14
		Anæsthesia.....	4	2500	0.32
		Final.....	10	3600	0.70
2	IV	Preliminary.....	3	2800	0.21
		Anæsthesia.....	2	2200	0.14
		Final.....	3	2600	0.30
3	V	Preliminary.....	3	3200	0.24
		Anæsthesia.....	1	1200	0.09
		Final.....	5	3000	0.45
	VI	Preliminary.....	2	2500	0.18
		Anæsthesia.....	1	1500	0.10
		Final.....	4	2800	0.40
	VII	Preliminary.....	2	2500	0.20
		Anæsthesia.....	2	2500	0.18
		Final.....	5	3000	0.45
	VIII	Preliminary.....	3	2800	0.27
		Anæsthesia.....	1	1500	0.11
		Final.....	13	3500	1.43
5	IX	Preliminary.....	6	2800	0.48
		Anæsthesia.....	3	2500	0.27
		Final.....	7	2900	0.63

TABLE XV.
Nitrogen Balances.

dog.	Experi- ment	PERIOD.	Length of period.	NITROGEN.							
				Income.	Outgo.					Gain or loss.	Average gain or loss per day.
				Food.	Urine.	Feces.	Hair.	Wash			
			days	grams.	grams.	grams	gms.	gms.	grams.	grams.	
1	I	Preliminary .	4	16.40	14.95	1.01	0.32	0.24	- 0.12	-0.03	
		Anæsthesia .	2	8.20	8.75	0.64	0.14	0.14	- 1.47	-0.74	
		Final.....	4	16.40	15.34	1.31	0.28	0.32	- 0.85	-0.21	
		Total.....	10	41.00	39.04	2.96	0.74	0.70	- 2.44	-0.24	
	II	Preliminary .	2	8.20	7.34	0.66	0.14	0.16	- 0.10	-0.05	
		Anæsthesia ..	2	8.20	8.40	0.71	0.16	0.14	- 1.21	-0.61	
		Final.....	5	20.50	19.87	1.50	0.30	0.35	- 1.52	-0.30	
		Total.....	9	36.90	35.61	2.87	0.60	0.65	- 2.83	-0.31	
	III	Preliminary .	2	8.20	7.54	0.60	0.12	0.14	- 0.20	-0.10	
		Anæsthesia .	4	16.40	19.01	1.22	0.28	0.32	- 4.43	-1.11	
		Final.....	10	41.00	43.07	3.49	0.80	0.70	- 7.06	-0.71	
		Total.....	16	65.60	69.62	5.31	1.20	1.16	-11.69	-0.73	
		Grand Total for Dog. No. 1	35	143.50	144.27	11.14	2.54	2.51	-16.96	-0.48	
2	IV	Preliminary .	3	15.09	13.94	0.88	0.27	0.21	- 0.21	-0.07	
		Anæsthesia .	2	10.06	9.91	0.56	0.18	0.14	- 0.73	-0.37	
		Final.....	3	15.09	12.99	0.83	0.27	0.30	+ 0.70	+0.23	
		Total.....	8	40.24	36.84	2.27	0.72	0.65	- 0.24	-0.08	

TABLE XV—CONTINUED.

Nitrogen Balances.

dog.	Experi- ment	period.	Length of period.	NITROGEN.						
				Income.	Outgo.					Average
				Food.	Urine.	Feces.	Hair.	Wash	Gain or loss.	gain or loss per day.
			days	grams.	grams.	grams.	gms.	gms.	grams.	grams.
3	V	Preliminary .	3	21.51	19.66	1.11	0.24	0.24	+ 0.26	+0.09
		Anæsthesia .	1	7.17	7.11	0.34	0.09	0.09	- 0.46	-0.46
		Final.....	5	35.85	30.61	1.70	0.45	0.45	+ 2.64	+0.53
		Total.....	9	64.53	57.38	3.15	0.78	0.78	+ 2.44	+0.27
	VI	Preliminary .	2	14.34	12.94	0.68	0.18	0.18	+ 0.36	+0.18
		Anæsthesia .	1	7.17	7.32	0.37	0.09	0.10	- 0.71	-0.71
		Final.....	4	28.68	26.18	1.48	0.36	0.40	+ 0.26	+0.07
		Total.....	7	50.19	46.44	2.53	0.63	0.68	- 0.09	-0.01
	VII	Preliminary .	2	14.34	13.29	0.74	0.18	0.20	- 0.07	-0.04
		Anæsthesia .	2	14.34	14.32	0.67	0.20	0.18	- 1.03	-0.52
		Final.....	5	35.85	31.33	1.76	0.50	0.45	+ 1.81	+0.36
		Total.....	9	64.53	58.94	3.17	0.88	0.83	+ 0.71	+0.08
	VIII	Preliminary .	3	21.51	19.42	1.05	0.30	0.27	+ 0.47	+0.16
		Anæsthesia .	1	7.17	7.08	0.33	0.09	0.11	- 0.44	-0.44
		Final.....	13	93.21	92.57	3.77	1.17	1.43	- 5.73	-0.44
		Total.....	17	121.89	119.07	5.15	1.56	1.81	- 5.70	-0.34
		Grand Total for Dog No. 3	42	301.14	281.83	14.00	3.85	4.10	- 2.64	-0.06
5	IX	Preliminary .	6	37.59	34.22	1.74	0.54	0.48	+ 0.61	+0.10
		Anæsthesia .	3	18.80	19.00	0.81	0.24	0.27	- 1.52	-0.51
		Final.....	7	43.86	41.38	1.96	0.56	0.63	- 0.67	-0.10
		Total.....	16	100.25	94.60	4.51	1.34	1.38	- 1.58	-0.10

BACTERIAL GROWTH AND CHEMICAL CHANGES IN MILK KEPT AT LOW TEMPERATURES.

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*(Contribution from the United States Department of Agriculture, Bureau of
Chemistry.)*

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That the growth of organisms in milk is delayed by cold and that there is, consequently, a retardation of those processes which finally render the milk unfit for food are facts which have long been made of practical value in milk preservation. In comparatively recent years the large cold storage warehouses of our cities and towns have offered better facilities for keeping such readily altered products at temperatures so low that the ordinary course of bacterial decomposition and putrefaction is entirely changed, though, as we shall see, the growth of certain kinds of organisms is by no means stopped, but rather so slowed that weeks and months may be required for them to gain great headway.

It is popularly supposed that when milk is kept very cold bacterial life is quiescent. For the bettering of the milk supply of our large cities, or whenever any length of time elapses between the drawing of the milk and its consumption, we urge, and quite properly, that the milk be iced constantly, and that its temperature shall never be allowed to exceed 50° F. To the lack of ice in milk transportation we trace a very great proportion of the bacterially dirty milk of cities, and no single factor in dairying is so largely responsible for multiplicity of numbers of organisms in city milk as is the hot car or wagon, or platform where the milk may spend hours on even the hottest of summer days.

The rate at which organisms in milk multiply at comparatively elevated temperatures has been the subject of numerous studies by many investigators. That when ice packed the rate is slow—so slow that from the commercial standpoint it is quite disre-

garded—is proven by careful milk dealers and dairymen everywhere, and laboratory studies have amply demonstrated that clean milk, kept cold, is more apt to lose organisms during the first twenty-four to forty-eight hours than to gain them.

Conn and Esten¹ report three experiments where milk was kept at 1° C. They found that scarcely any bacterial development took place for from six to eight days, after which time there was a steady increase until very great numbers were present, though the usual lactic acid organisms were not in the majority, therefore the milk did not curd. They also found a comparatively large number of gelatin liquefying organisms and of the “neutral” milk organisms; that is, bacteria which produce neither alkali nor acid. In the summary they state that “Milk is not necessarily wholesome because it is sweet, especially if it has been kept at low temperatures. At the temperature of an ice chest milk may remain sweet for a long time and yet contain enormous numbers of bacteria among which are species more likely to be unwholesome than those that develop at 20° C.” The latter temperature the above authors proved to be most favorable to the development of the *Bacterium lactis acidi*, the products of which cause curdling when excessive and are, in themselves, harmless.

In ordinary milk commerce the consumer receives the milk when it is from twenty-four to forty-eight hours old. In large cities especially it is rarely delivered before it is at least a day old and, in the heated term, the dealer generally depends upon ice box facilities as a means of preservation. However, he does occasionally store at low temperatures for a considerably longer period. When a shortage of milk is feared because of transportation difficulties, as in heavy snow storms, or to save up milk and cream to meet the increased demand for ice cream on July 4, he may use the cold storage warehouse, or simply deep troughs in which, buried in ice, the cans, especially those for ice cream, are kept for weeks.

The cold storage of milk and cream for use on ocean voyages is now accepted as a matter of course. So confident of successful preservation of milk and cream has the commissariat of the trans-

¹ Storr's Agricultural Experiment Station Report of 1904.

atlantic liners become that the vessels are provisioned for not only the single, but for the *round* trip. Hence, on the homeward bound voyage the milk served is more than two weeks old.

What would seem to be the extreme of milk refrigeration has been reached at Saint-Laurent-en-Champsaur (Haute-Alpes) where milk is frozen at a temperature of -5° C., the resulting friable mass reduced to a powder and so shipped to Marseilles which it reaches in a frozen condition.¹

Such facts of themselves made a study, both bacteriologically and chemically, of milk kept at low temperatures of sanitary importance. In the general agitation for the icing of milk and the refrigerating plants that some of the larger milk dealers are now installing we may encourage conditions which, while to be in every way commended for short periods of service, should not be advocated for the long continued preservation of milk.

Aside from the importance of the growth of organisms and the chemical changes taking place as affecting the question of milk *per se*, we have in this substance an ideal medium for the growth of many kinds of bacteria. Its constituents include representatives of the great classes of food components—fats, proteids, carbohydrates and mineral salts—and its physical characteristics make it possible to manipulate it with but a minimum of experimental error. The fact that fresh, raw milk has also the essentials of vitality, as has the recently excised flesh, and that its serum is comparable with that of the blood, gives a broader significance to its study than appears at first sight.

SOURCE AND QUALITY OF MILK. These experiments have been conducted upon the purest milk obtainable and also upon the ordinary market milk, the two being run side by side wherever possible, and the results compared. The high-grade milk was procured from a dairy furnishing "certified milk." The organisms are ordinarily below 2000 per cc. even when the milk is twenty-four hours old. That used in these experiments reached us when six hours old. It was shipped directly to the cold storage warehouse in a special can holding ten quarts, made with a mushroom lid which was sealed and carefully protected from bacterial infection. The initial count was taken immediately upon its receipt.

¹ *L'hygiène de la viande et du lait*, i, p. 137, 1907.

Both veterinary and laboratory supervision of the herd furnishing this milk is exceedingly minute, the cattle being clinically inspected at short intervals and each cow in the herd having her milk examined microscopically once a week for the presence of indications of udder inflammation. Every step in the production and collection of the milk is conducted with the utmost cleanliness and with every precaution against contamination.

The market milk, on the other hand, was procured from an ordinary dairy of medium grade. It was a very fair sample of the milk which is usually sent to cities. It was shipped however in one of the special cans described above and was delivered, as was the clean milk, directly at the cold storage warehouse. It was, therefore, put into cold storage twenty-four hours before it would have reached the consumer had it gone through the hands of the dealer as such milk generally does. The initial count of this milk varied between wide limits as the experiments show.

STORAGE CONDITIONS. Since it was desired in this investigation to study the effect of temperatures considerably lower than that obtained in the ordinary ice box, and also of a wider range of temperature than could be maintained in an ice and salt cooler, it was decided to store the experimental cans and flasks in a refrigerating warehouse where the various conditions necessary might be maintained. To this end a room was especially prepared for the reception of this milk. It was supplied with new wood work, rendered thoroughly clean and fresh and nothing has been stored in it except the milk under observation.

Chilling is accomplished by means of a calcium chloride system. The entrance to the room is through heavily insulated doors opening into an outdoor court. The temperature of the room is taken eight times during each twenty-four hours by a man especially appointed for that purpose, and as the only entrance is through this outer door a certain amount of fresh air is admitted eight times, at least, during each twenty-four hours.

Ordinarily the variation of temperature does not exceed 1.5° F. A few times, however, but for periods of very short duration, the temperature has varied within a limit of 3° F. That is, if a temperature of 30° F. was desired the maximum reading for the entire experimentation period would not exceed 31.5° F. nor the minimum fall below 28.5° F.

EXPERIMENTAL METHODS. The lower temperatures used in this work caused the milk to become, finally, a mass of small particles of ice, loose in the middle of the can but an adherent layer an inch or more in thickness about the sides. To obtain homogeneous samples of this semisolid mass necessitated the use of the following device: Copper cylinders were made six inches long and two inches in diameter, mounted on long copper handles with an opening in the bottom of the cylinder which could be closed with a copper cork-shaped plug, also mounted on a stout copper handle. All of the seams in this piece of apparatus were brazed; hence hot air sterilization could be resorted to with safety. The stout plug and handle could be used to dislodge the adherent ice around the sides of the cans and also, by stirring, to incorporate it as well as possible with the looser particles. This method, and also vigorous shaking of the can, made a fairly homogeneous mass into which the copper cylinder was plunged. When full the plug was inserted and the apparatus was then placed over the neck of a wide mouthed sterile flask, when the removal of the stopper allowed the contents to drop into the flask with a minimum amount of air contact.

Of course as many precautions as possible were taken to prevent the contamination of the milk in the can, such, for instance, as care on the part of the experimenter not to lean over the can while the lid was off and to wrap the arm holding the sampling apparatus in a sterile towel before stirring or removing the sample. Where the temperature was sufficiently high to prevent the formation of solid particles, samples were removed with an ordinary sterile glass pipette.

The small flasks containing the samples to be examined were conveyed with all promptness to the laboratory. The length of time consumed between leaving the cold storage warehouse and arrival at the laboratory is from five to seven minutes. Hence, even in warm weather the change in the temperature of the milk was so slight as to preclude the likelihood of either bacterial or chemical changes of importance.

BACTERIOLOGICAL TECHNIQUE. The number of organisms in the milk was found by plating in suitable dilution and counting the colonies in the usual way. In order to have some idea of the qualitative, as well as the quantitative relation of these

organisms plates were made on several different kinds of media as follows: A plain nutrient agar was used for the total count. A lactose agar, containing sufficient litmus to color it a clear blue, was used to pick out, more especially, the acid-forming organisms; and a lactose-litmus gelatin served for the detection of those organisms which form proteolytic enzymes.

It has been deemed better in the progress of this work to use these various media rather than the litmus-lactose gelatin alone, as was done by Conn and Esten,¹ for the reason that we desired to watch the development of these organisms at various temperatures; and we also found that different degrees of dilution were preferable for the counting of the different classes of organisms. For example, it was better to count the gelatin liquefiers in much greater dilution than the acid formers, since a few rapid liquefiers will often obliterate all of the other organisms before their chemical relations are sufficiently well marked to admit of their identification. Occasionally the total gelatin count at 20° ran notably higher than the total lactose agar count made at the same temperature. When such a difference was observed the higher gelatin count was accepted as the total for that temperature.

Considerable difficulty was encountered in making dilutions of such strength that the plates would contain a desirable number of organisms, that is, except in the case of the liquefiers, from 200 to 300 per plate, because there is a great variation in the rapidity of the multiplication of different samples, and also, as will be seen from the appended tables, the number of organisms developing at the different temperatures varies widely. Hence, a great many plates for each temperature and on each nutrient substance had to be made. Even with this precaution there are breaks in the results, due to inability to count accurately either the total number or the classes of organisms present.

In the early part of this work the agar plates were grown at 37° C. and the gelatin plates at 20° C., as is customary in determining the number of organisms in milk. Later on, for reasons which will appear presently, three sets of plates were made: one for the incubator, one for the 20° box and one for the cold storage

¹ *Bulletin of Storr's Experiment Station, 1904.*

temperature at which the milk had been kept. The plates in the incubator were usually removed at the end of twenty-four hours and kept three days at room temperature before counting. Such a procedure, especially where large numbers of organisms are present, results in cleaner, sharper colonies than develop when the plate is kept for the entire length of time at 37°C . The numerical results, on the other hand, do not vary. The plate at 20°C . required about five days to develop except those studied especially for liquefiers, which frequently had to be counted in from twenty-four to forty-eight hours, while those in cold storage needed from a month to six weeks before showing satisfactory colonies.

In the analytical study of the bacteriological results obtained in this investigation the number of organisms developing at 20°C . has been accepted as the total. This was decided upon because in many cases, the numbers growing at this temperature were actually the higher and it seemed to afford the best basis for comparison. Undoubtedly, however, a study of the number of colonies developing at various temperatures between the three chosen would show in some of the experiments a greater number of bacteria than we have reported. Because the numerical variation depending upon the incubating temperature at which the plates were kept was so wide suggests that a closer study of this phase of the work would be very interesting but the great multiplication of plates that it would involve rendered such a procedure impossible while prosecuting the other lines of work here mapped out.

In the beginning of the work of identification of the various species of organisms the subcultures were grown at the temperature at which the plate colony had originally developed. For those species which grew in cold storage, however, the characteristic reactions were often so altered that naming the organism was impossible and subcultures had to be grown at 20°C . and 37°C ., after which their classification proceeded along routine lines.

Such an observation, in the light of the study of the chemical changes of foodstuffs in cold storage, is of considerable interest, and it is proposed to develop this line of work in the future.

CHEMICAL TECHNIQUE. Coincident with the bacteriological study of these samples of milk there was made a chemical analy-

sis, more especially of the changes occurring in the proteid, as the period of storage advanced. On the fresh sample of milk the amount of fat was determined. But this constituent was not further studied.

The acidity, expressed in the number of cc. of $\frac{N}{10}$ sodium hydrate required for each 100 cc. of the milk, was determined at short intervals. Phenolphthalein was used as an indicator. The methods of analysis for the determination of the various proteid constituents are those adopted by the Association of Official Agricultural Chemists, and are, very briefly, the total nitrogen according to Gunning's modification of the Kjeldahl method, the separation and estimation of casein, albumin and syntonin and the cascoses, by the methods of Van Slyke and Hart,¹ and the peptones by the tannin-salt method as modified by Bigelow and Cooke.²

The portions for analysis were of course weighed, not measured, and by the aid of a vessel of water they were quickly brought to room temperature before weighing.

As the samples of milk gained in the number of organisms, and also in acidity, it was found exceedingly difficult to separate the casein cleanly by the acetic acid method. The full portion of acid recommended by Van Slyke and Hart, for use with fresh milk, cannot be used in these old milks since the natural acidity is so high that, combined with the added acid, it causes too great an excess to permit of a perfect precipitation. Hence it was found necessary to add acetic acid from a burette drop by drop, keeping the sample at 40° C. until a clean flocking of the casein took place. It is necessary also, especially in these old samples, to boil briskly and for a considerable length of time, the filtrate from the casein, in order to perfectly precipitate the albumin and syntonin,

The peptones have not been estimated directly. That such, however, are produced is indicated by the difference between the total nitrogen found and the sum of the nitrogen of the various nitrogeneous compounds determined.

The results, both chemical and bacteriological, so far as they are expressed by figures, are set forth in the various tables which follow. The proteids, as specified in the different columns are

¹ *New York Experiment Station Bulletin*, No. 215.

² *Journ. of the Amer. Chem. Soc.*, xxvii, p. 1485, 1906.

represented by the amounts of nitrogen expressed as the percentage of the milk taken. The figures in *italics*, below the actual percentage quantity, show the relation which it bears to the total nitrogen content of the milk.

The number of organisms per cubic centimeter, as found under the conditions described, are given in plain type. In the column devoted to the total number of organisms the *italics* indicate the relative proportion of bacteria, taking the development at 20° C. as the standard, which was found at 37° C. and the temperature at which the milk was stored, respectively. In the columns giving the acid forming and liquefying organisms the *italics* indicate the relation which they bear to the whole number growing at the temperature specified.

Experiment I, March 16, 1906.

Two cans of milk, holding ten quarts each, were placed in storage at a temperature of -1.67° C. to -0.55° C. One contained clean milk; the other ordinary market milk. Both cans were filled at their respective dairies and were received at the cold storage warehouse when about six hours old. They were examined immediately bacteriologically and, thereafter, at intervals of one week until by odor, taste or appearance, the milk was judged unfit for use. The first count of the number of organisms showed the following conditions:

	Colonies on agar at 37° C.	Acid forming colonies.	Liquefying colonies.
Clean milk.....	1,976	1,090	200
Market milk.....	15,956		1,150

Expressed in percentages we find that of the whole number the clean milk had 10.1 per cent liquefiers and 55.2 per cent acid formers while the market milk had 7.3 per cent liquefiers. In the latter the plates for acid formers were too thickly sown to differentiate colonies.

At the expiration of the first week, when the examination was again made, and the plates incubated at the temperatures already indicated, it was found that those grown at 37° C. gave markedly fewer colonies than did those grown at 20° C. So striking was this difference that laboratory errors were taken for granted and

other plates were made with fresh culture media and extra care, but the results were the same. This series of plates when grown at 20° C. gave a very decided increase in the number of the organisms while similar plates at 37° C. were almost sterile.

Three weeks after the first count was made the following results were obtained:

	Colonies on agar at 20° C.	Liquefying colonies.
Clean milk.....	94,250	13,300 = 13.0 per cent.
Market milk.....	11,420,000	760,000 = 6.1 " "

In the market milk the number of organisms steadily increased until at the end of five weeks they had reached the high numbers indicated below.

Colonies on agar at 20° C.	Acid forming colonies.	Liquefying colonies.
376,000,000	5,000,000 = 1.3 per cent.	13,600,000 = 3.6 per cent

At this time there was a slight odor to the milk. It did not curd even on boiling, and in spite of the enormous number of organisms that were present would have passed muster in many a household. After this time, however, there was a gradual numerical decline as the organisms of putrefaction got under way until, at the end of the eighth week, the count was:

Colonies on agar at 20° C.	Liquefying organisms.
99,000,000	5,700,000 = 5.7 per cent.

The odor was decidedly that of putrefaction. Heating caused the milk to curd.

The clean milk of this same experiment preserved both its tastelessness and lack of odor at the end of the sixth week, when the count showed:

Colonies on agar at 20° C.	Liquefying colonies.
137,000,000	3,000,000

At the end of the eighth week there was a slight odor such as is found in stale milk which is overgrown with organisms. However, it was not sour and did not curd even on boiling. Only a very fastidious person would have passed this milk by as unfit for food and it would have been perfectly acceptable in the baker's

shop or the kitchen. The number of colonies showed the organisms to be:

Colonies on
agar at 20° C.

144,000,000

Liquefying colonies.

2,700,000 = 1.8 per cent.

This experiment, though incomplete, and made simply as a preliminary to pave the way for future work, is still very largely in accord with the work done later and more accurately. The results are therefore included in this report.

Experiment II, June 6, 1906.

This experiment was made under the same conditions as those in Experiment I, except that the experience gained while working on Experiment I gave us a greater opportunity to study the detailed changes which occurred in this second experiment. Instead of one sample of milk, however, two were run, side by side, a can of a very clean milk and one of the usual market quality, in order that a comparison of the course and extent of alteration might be made.

The examinations were conducted weekly for a period of six weeks and included in this case both the bacteriological and the chemical study of the two samples. At the expiration of the sixth week the clean milk showed a bacterial count of 588,000,000 organisms per cc. when the plates were allowed to develop at -1.67 to -0.55° C., and 48,500,000 when the plates were grown at 20° C. There was nothing about the milk to indicate this high bacterial content. It was perfectly odorless and its flavor was much better than that of the usual city milk though it was not, by any means, equal to the original flavor of the clean milk. There was nothing in its appearance nor its separation of cream to indicate that it was beyond the usual market age. Neither did boiling curd it, though the acidity was fairly high.

At the end of the fourth week the market milk had an unpleasant, rather bitter, taste, and the count at 20° C. was 195,000,000. There were no indications of curding. One week later the bitter taste was intensified and a very distinct, and unpleasant, odor had developed.

Tables I and II present in detail the results obtained both bacteriologically and chemically. Column one gives the total

number of organisms developing on the plates kept at 37°C ., 20°C . and -1.67°C . to -0.55°C . It will be observed that while the colonies growing at the low temperatures steadily increased throughout the experiment until very high numbers were reached, those growing at 37°C . increased very slowly in actual numbers; and in clean milk the proportion of colonies surviving at 37°C ., accepting the count at 20°C . as the total, was reduced from 88 per cent to 0.0079 per cent after three weeks, when it remained practically stationary for another week and then there was a sharp rise to 0.11 per cent.

In the market milk there was a similar marked decrease in the number of organisms growing at body heat, 67.5 per cent of all the organisms present surviving at the beginning of the experiment whereas, at the end of the third week, the proportion had fallen to 1.33 per cent of the total.

It is of interest, also, to note that in this market milk, as in the clean milk, there is a proportional rise in the organisms growing at 37°C . on the sixth examination, when putrefaction could be detected by the odor.

A very marked irregularity in the proportion of acid forming organisms present in the clean milk, at the different periods of the experiment, is indicated in column two. However, the inability to determine the exact proportion of these organisms in a number of instances, has made so many breaks in the report of the experiment that conclusions cannot safely be drawn from it.

In the market milk, after the second week, the proportion of acid forming organisms developing at 37°C . is strikingly high and continues so to the end of the experiment. The proportion of these organisms developing at 20°C ., in the beginning of the experiment, was about the same in number as those growing at 37°C ., but after one week in cold storage there was a very decided decrease relatively. At the end of the third week the acid formers had increased again to about their original proportion and, at the close of the experiment, they reached a total of 59.2 per cent—a considerably higher number than that present at the beginning of the experiment. The number of acid formers developing at the temperature of the cold storage warehouse was generally low until the fourth week and by the end of the fifth week it had reached 39.4 per cent.

While the liquefiers in both of these experiments showed a very marked increase in numbers we had not at this time acquired sufficient skill in the plating of these organisms to obtain more than a few scattered reliable counts, which are found in column three of Tables I and II.

It would seem desirable to have some idea of not only the number of organisms present, and their behavior according to a general chemical classification, but also to know at least the predominating species developing at the various temperatures. Accordingly plates from Experiment I have been examined at periods indicated in Table III, and the species there listed have been determined. It will be noted that the fresh milk—both the clean and the market product—contained a considerable number of varieties of the ordinary milk organisms when first examined. That the market milk is in this case, as well as in the others cited, poorer in species is due, probably, to the warring elements and the consequent survival of greater numbers of fewer varieties. The majority of these found 37° C. the most favorable temperature.

The initial plates made for -1.67° C. from the clean milk were, unfortunately, not sufficiently concentrated to catch the organisms surviving. Their number, however, was less than one per cubic centimeter since this amount was divided amongst the various plates incubated and all were sterile.

At the end of the second week in cold storage the predominating species in the clean milk developing at 20° C. were two in number, while at the temperature of the original storage of the milk only one organism developed, namely, *B. solitarius* Ravenel. The market milk at this time had also one species developing well at low temperatures, namely, *B. formosus* Ravenel.

The number of species as storage continues, markedly diminishes so that by the end of the fifth week but three in the clean milk—*B. formosus*, *B. subocraceum* and *Sarcina subflava*—remained from the seven species which were plentiful in the beginning and which developed at 37° C. In the market milk, at this time, we also find three well represented varieties, namely, *Sarcina lutea*, *Bac. aerogenes* and *B. formosus*.

In the clean milk the *B. solitarius* and *B. formosus* were most noticeable on the low temperature plates while in the market

TABLE I.
Clean Milk. Storage Temperature, -1.67° to -0.55° C. (Experiment No. II.)

No. of days.	Organisms developing at A 37° C. B 20° C. C -1.67° to -0.55° C.	Acid formers developing at A 37° C. B 20° C. C -1.67° to -0.55° C.	Liquefying organisms developing at 20° C.	Acidity $\frac{1}{2}$ NaOH per 100 cc.	Casein nitrogen. per cent.	Albumin and syn- tonin nitrogen. per cent.	Glucose nitrogen. per cent.	Amino nitrogen. per cent.	Total nitrogen.	Difference between total nitrogen and that estimated.
June 6, 1906	A 800 88%	300 37.5%	300 9.33%	15	0.404 81.25	0.032 5.25	0.069 11.34	0.0211 3.47	0.608	+0.008
	B 900	400 44.4%								
	C 100 1.11%									
7 days....	A 2,000 33%		36,000 33%	22	0.304 60.0	0.032 5.25	0.025 4.1			
	B 6,000									
	C 1,000 16.6%									
14 days ...	A 2,000 1.2%	29,000 18.5%			0.308 60.6	0.030 4.9	0.033 5.4			
	B 156,000	33,000 34.7%								
	C 95,000 60.8%									
21 days ...	A 700 0.0079%	700 100%			0.267 43.9	0.0324 6.3	0.0428 7.0			
	B 8,800,000	4,750,000 53.9%								
	C 8,200,000 93.1%									
28 days ...	A 5,000 0.0086%	18,000,000 62.1%	4,200,000 12%	39	0.216 36.6	0.0436 7.1	0.0260 4.4			
	B 34,500,000	14,500,000 18.1%								
	C 80,000,000 231.8%									
35 days ...	A 56,000 0.11%	9,200,000 18.9%			0.282 46.5	0.028 4.6	0.0152 2.6			
	B 48,500,000	25,000,000 4.2%								
	C 588,000,000 1212.3%									

TABLE II.
Market Milk. Storage Temperature, -1.67° to -0.55° C. (Experiment No. II.)

No. of days.	Organisms developing at A 37° C. B 20° C. C -1.67° to -0.55° C.	Acid formers developing at A 37° C. B 20° C. C -1.67° to -0.55° C.	Liquefying organisms developing at 20° C.	Acidity % NaOH per 100 cc.	Casein nitrogen. per cent.	Albumin and syn- tonin nitrogen. per cent.	Caseose nitrogen. per cent.	Amino nitrogen. per cent.	Total nitrogen.	Difference between total nitrogen and that estimated.
June 6 1906	A 50,000 B 67,500 C 13,000	A 7,000 14% B 11,000 14.8% C 17.5%	2,700 5.5%	17						
7 days	A 42,000 B 2,230,000 C 125,000	A 2,000 4.7% B 40,000 1.79% C 35,000 68.0%	145,000 6.5%	17	0.254 44.71	0.0244 4.29	0.043 7.57		0.568	-0.249 48.83%
14 days ...	A 1,195,000 B 43,700,000 C 24,600,000	A 11,000 0.92% B 8,150,000 18.6% C 60.2%	650,000 1.4%	17	0.210 36.9	0.0298 5.2	0.045 7.9			-0.2832 49.86%
21 days ...	A 1,150,000 B 80,000,000 C 79,500,000	A 685,000 59.6% B 15,500,000 18.0% C 12,000,000 16.8%	*	19	0.212 37.3	0.0296 5.2	0.047 8.2			-0.2886 50.80%
28 days ...	A 2,700,000 B 105,000,000 C 185,000,000	A 200,000 74.0% B 115,000,000 68.9% C 64,000,000 94.6%	*	19.5	0.210 36.9	0.0293 5.1	0.0466 8.7			-0.2791 49.13%
35 days ...	A 2,500,000 B 270,000,000 C 192,500,000	A 160,000,000 6.92% B 76,000,000 30.4% C 71.2%	*	20	0.197 34.63	0.0424 7.48	0.0335 5.80	0.200 45.77		-0.0351 12.66%

* Too many colonies to count.

TABLE III.

*Predominating Species of Organisms in Experiment No. II.**

No. of days.	Temperature.	Clean milk.	Market milk.
June 6, 1906	37° C.	<i>Mic. citreus</i> Chester. <i>Mic. tenacatis</i> Chester. <i>Bac. ferrugineum</i> Dyar. <i>Bac. fluorescens</i> Zimmerman. <i>Oidium lactis</i> . <i>Saccharomyces</i> . A micrococcus, not classified.	<i>Mic. citreus</i> Chester. <i>Saccharomyces</i> . A white fungus (?). An orange micrococcus.
	-1.67° to -0.5° C.	No growth.	Only one organism, namely, <i>Sarcina lactis</i> .
7 days	20° C.	<i>Sarcina lutca</i> (?) Chester. <i>Mic. aurantiacus</i> Cohn. <i>B. solitarius</i> Ravenel.	<i>Mic. rosus</i> Chester. <i>B. Lesagei</i> (?) Chester. <i>B. formosus</i> Ravenel.
	-1.6° to -0.5° C.		
21 days	37° C.	A spore former and an unclassified micrococcus.	
	20° C.	<i>Sarcina subflava</i> . <i>B. formosus</i> .	<i>Sarcina subflava</i> . <i>B. formosus</i> . <i>B. aerogenes</i> . <i>B. haematoides</i> . <i>B. solitarius</i> Ravenel.
	-1.6° to -0.5° C.	<i>B. solitarius</i> Ravenel.	
28 days	37° C.	<i>Mic. citreus</i> Chester.	<i>Sarcina subflava</i> .
	20° C.	A small yeast. <i>B. formosus</i> .	<i>B. cloacæ</i> . <i>Oidium lactis</i> . <i>B. coli</i> Chester. <i>B. solitarius</i> Ravenel.
	-1.67° to -0.5° C.	<i>B. solitarius</i> .	
35 days	37° C.	<i>B. formosus</i> . <i>B. subocraceum</i> . <i>Sarcina subflava</i> . <i>B. formosus</i> .	<i>Sarcina lutca</i> . <i>Bac. aerogenes</i> . <i>B. formosus</i> . <i>Mic. citreus</i> . <i>Bac. aerogenes</i> . <i>Bac. aerogenes</i> or <i>B. coli</i> . <i>B. solitarius</i> Ravenel.
	20° C.		
	-1.67° to -0.5° C.	<i>B. solitarius</i> .	

* For the classification of the organisms in these Experiments I have to thank Dr. George W. Stiles of this Bureau.

milk, though these were found almost exclusively in the early stages of storage, they were finally mixed with organisms of the coli group.

CHEMICAL. The chemical findings in Experiment II are noted in columns 5 to 10, respectively, of Tables I and II. The most marked chemical change observed on keeping this milk is its loss in casein nitrogen. The clean milk at the end of the fourth week has but 35.5 per cent of the total nitrogen in the form of casein, there having been a gradual loss from the initial analysis, which showed 81.25 per cent of the nitrogen present bound in the casein.

In the market milk there is also a decided digestion of the proteid, resulting in a greater degradation of nitrogen finally than is seen in the clean milk. Unfortunately, the initial analysis in this sample was lost, but a comparison of those made at the end of the first week shows less casein for the market than for the clean milk.

This digestion of the casein proceeds, apparently, to amino-acids, and as there is generally a considerable discrepancy between the sum of the nitrogen determined and the total nitrogen it is likely that peptones are also produced.

Experiment III, October 4, 1906.

Experiment III was made on market milk kept at a temperature of -1.67° C. to -0.55° C. Its container was a can such as has previously been described. The initial count of this milk, as will be seen from Table IV, was decidedly high and the incubator count was 380 per cent higher than the count of the same milk when the plates were kept at 20° C., while the organisms developing at the cold storage temperature were but 14 per cent of those developing at 20° C. Subjecting the milk to the low temperature of the storage warehouse, however, showed at the end of the first week a very rapid rise in the number of organisms developing at 20° C., which rise continued until the end of the fifth week when it reached the sum of 1,000,000,000 organisms. The organisms developing in cold storage also rose rapidly and reached their maximum at the end of the fifth week when they were 1,090,000,000, a somewhat greater number than developed at 20° C.

The incubator counts of this milk show a very decided irregularity due, probably, to the rise and fall of certain definite species. The minimum count was reached at the end of the fourth week, when the total number had fallen to 850,000, which represented 0.12 per cent of the 20° count,

During the final week that this milk was studied there was a slight falling off in the number of organisms developing at 20° and at cold storage temperature, -1.67 to -0.55 , and the incubator count remained about equal to that found the week previously.

The acid formers developing at ice box temperature show a very marked decrease, proportionally, at the end of the second week, after which time they recovered the ground lost and when the total count reached the maximum they were, approximately, in their original proportion. During the final week of the cold storage they were again reduced to comparatively few. The number of plates counted at 37° C. for acid formers were too few to make deductions advisable.

The liquefying organisms in this experiment were decidedly high in the beginning, showing 25.5 per cent of all present to be of this character, while the total number increased so that at the end of the second week there were present 10,000,000 liquefiers. The proportion of the whole number was but 5.2 per cent and at the end of the following week (the third) they were reduced to 1.6 per cent of the total, though the actual number of organisms of this type was 8,000,000. In the fourth and fifth weeks they increased both proportionately and in total numbers, their maximum count coinciding with the maximum count of the organisms in the fifth week, when there were 70,000,000, that is, a proportion of 7 per cent.

The taste of this milk in the beginning was that of the ordinary market milk, having a decidedly "cowy" flavor, which is readily accounted for when one considers that it had a count of over two million organisms per cubic centimeter, and 25.5 per cent of these bacteria liquefied gelatin. This taste did not materially change for three weeks. The fourth week showed it to be slightly bitter; the fifth week developed rather an unpleasant odor, and at the end of the sixth week, though the milk was not sour and did not curd even on heating, it had an odor of putrefaction and would

TABLE IV.
Market Milk. Storage Temperature, -1.67° to -0.55° C. (Experiment No. III.)

No. of days.	Organisms* developing at A 37° C. B 20° C. C 0° C.	Acid formers developing at A 37° C. B 20° C. C 0° C.	Liquefying organisms developing at 20° C.	Casein nitrogen. per cent.	Albumin and syn- tonin nitrogen. per cent.	Casease nitrogen. per cent.	Amino nitrogen. per cent.	Total nitrogen.	Difference between total nitrogen and that estimated.
Oct. 4, 1906	A 2,736,000 B 350,000 C 103,800	150,000 5.4% 150,000 20.8% 4,880 4.7%	181,000 25.5%	0.430 66.6	0.0745 11.6	0.1051 16.2	0.016 7.12	0.646	±0.000 1.39%
7 days	A 1,400,000 B 12,500,000 C 6,600,000	2,000,000 22.4% 40,000 60%	1,000,000 12.4%	0.420 65.0	0.1227 18.9	0.0784 12.1	0.0503 9.1		0.000
14 days ...	A 50,016,000 B 190,000,000 C 142,000,000	0,000,000 3.1% 50,000,000 39.8% 1,000,000 18%	10,000,000 6.2%	0.364 56.3	0.107 16.6	0.116 17.9	0.042 6.6		
21 days ...	A 5,500,000 B 405,000,000 C 268,000,000	8,000,000 1.6% 30%	8,000,000 1.6%	0.368 56.9	0.180 27.8	0.141 21.3	0.072 11.1		-0.003 0.030%
28 days ...	A 3,875,000 B 980,000,000 C 531,500,000	985,000 25.4% 270,000,000 27%	30,000,000 4.4%	0.312 48.2	0.113 17.4	0.091 14.0	0.125 19.3		-0.001 0.015%
35 days ...	A 3,000,000 B 916,000,000 C 607,500,000	250,000 7.6% 4,200,000 0.45%	70,000,000 7%	0.271 41.9	0.0882 13.6	0.130 20.1	0.111 17.1		-0.037 5.72%
42 days ...	A 3,000,000 B 916,000,000 C 607,500,000	250,000 7.6% 4,200,000 0.45%	70,000,000 7%	0.271 41.9	0.0882 13.6	0.130 20.1	0.111 17.1		

in all probability, have been discarded by a housewife though it would, in all likelihood, have been used for commercial purposes.

It will be noticed from the chemical study of this milk that it was probably rather stale in the beginning, only 66.5 per cent of total nitrogen being in the form of casein. There was present over 7 per cent of amido-acid nitrogen. The casein nitrogen continued to decrease quite steadily throughout the experiment. The caseose nitrogen generally gained from week to week, while the amino nitrogen of the last three examinations showed a very marked rise, and at the end of the sixth week, when putrefaction was under way, there was a decided discrepancy between the total nitrogen and the sum of the nitrogen determined, 0.037 per cent being referable to degradation products lower than caseoses.

The determination of the acidity of this sample of milk when fresh, when just removed from cold storage and after keeping at ice box temperatures for varying lengths of time, is given in Table V. The increase in acidity at the end of the second week is but slight. Between this time and the end of the fourth week there is a very decided rise, more than enough to curd the milk under ordinary conditions, and the number of bacteria, 680,000,-000 per cc., would indicate, also, that curdling should take place.

TABLE V. ACIDITY,
Market Milk. Storage Temperature.

No. of days in C.S.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.
	cc.		cc.		cc.		
0	14.8						
7	15						
14	19.3						
21	24.5	1	45.8	2	55	4	curd.
28	50	1	53.3	9	84.8	10	curd.
35	49.2	2	72.8	9	curd		
42	56.4	1	56	4	curd.		

A portion of this milk, taken out of cold storage at the end of the third week and kept for one day in the ice box, showed a marked gain in acidity; at the end of two days a still greater

gain but no curding, and this did not take place until the milk had been kept for four days at the temperature of the usual house refrigerator. At the end of the fourth week another sample subjected to the same conditions, held its own for nine days at the end of which time 84.8 cc. of $\frac{N}{10}$ alkali were required to neutralize it. On the tenth day it curded spontaneously.

There was apparently no change in acidity during the fifth week in cold storage, though the bacteria increased about 4,000,000 to each cc. This sample curded in nine days in the ordinary ice box. Probably because of the growth of putrefactive organisms during the sixth week there was a comparatively slight increase in the acidity, and only four days were required in the ice box to curd it.

There is a most striking difference between the behavior of this milk and milk as ordinarily kept, in so far as the acid figures are concerned. The organisms are numerous enough to cause curding and the number of acid forming organisms is fairly high. That they are functioning in the production of acid is proven by the titration results given in Table IV, but apparently the other organisms present have so modified the course of the reaction that curding is delayed for an unusual length of time.

The three foregoing samples of milk were kept at a temperature of 29° to 31° F., a degree of cold that, when maintained for several weeks, would seem to be sufficient to cause the fluid to solidify. This phenomenon, however, did not occur. For two weeks the fluid was the normal consistence and even around the edges of the can particles of ice had not formed. After this, on shaking, a few ice crystals were dislodged from the outer portion of milk, and these gradually increased in number until, at the close of the experiment, the entire can of milk was a semi-solid mass of small crystals. These, if undisturbed, would have collected into a firm layer an inch or so in thickness around the sides of the can, but, because of obtaining homogeneous samples for analysis, frequent stirring was resorted to and a layer of ice prevented.

Experiment IV, Market Milk put in Cold Storage November 21, 1906, at a Temperature of 0° C.

A statistical report of this experiment is given in Tables VI, VII and VIII. The initial count of milk showed that it was a fairly clean market milk containing, at 37° C., 118,333 organisms per cc., somewhat more than developed at 20° and only 68 per cent of which developed in cold storage. As in Experiment III there is here a steady rise in the number of organisms developing at 20°, the final examination at the end of the fifth week showing over 3,000,000,000 as the total number. Apparently, for the organisms in this sample, higher temperatures were the more favorable since the organisms developing at 0° C. never exceeded those developing at 20°, though in several counts they ran very close. Their maximum was reached at the end of the fourth week when 1,357,000,000 bacteria were present. The organisms developing at incubator temperatures gained steadily after the end of the first week, at which time they had dropped to 36,700. Indeed, the incubator counts here are unusually high throughout the experiment, the final count being 3,875,000,000, which is the maximum count at any temperature for this experiment. There has been noticed in previous experiments and also in this a tendency toward a rise in the numbers of high temperature organisms at the very last stages of the keeping of the milk.

The study of the growth of the acid formers would indicate that the natural acidifying organisms of milk were present here originally in considerable proportion since 29 per cent developed at 20° C., while only 12 per cent developed at 37° C., and throughout the entire experiment this high proportion holds. However, the acid formers developing at 37° C., also show a decided rise. This may be accounted for by the presence of certain intestinal acid formers, as *B. cloacæ*.

The gelatin liquefying organisms, so far as their percentage relations are concerned, are considerably higher at the beginning of the experiment than at any other time during its course. In actual numbers, however, they reach at the end of the fourth week 93,000,000 per cc., and at the end of the fifth week—when the milk would have been discarded by many—they were not much higher, only 110,000,000.

TABLE VI.
Market Milk. Storage Temperature, 0° C. (Experiment No. IV.)

No. of days.	Total number of organisms developing at A 37° C. B 20° C. C 0° C.	Acid formers developing at A 37° C. B 20° C. C 0° C.	Liquefying colonies developing at 20° C.	Caseln nitrogen. per cent. 0.420 75.3	Albumin and syn- tubin nitrogen. per cent. 0.0845 14.8	Caseose nitrogen. per cent. 0.063 9.3	Amino nitrogen. per cent. 0.0095 1.0	Total nitrogen.	Difference between total nitrogen and that estimated.
Nov. 20, 1906	A 118,333 166% B 102,000 C 70,000 68.0%	15,000 12.0% 30,000 29.1% 1,300 1.7%	31,000 30.5%	0.420 75.3	0.0845 14.8	0.063 9.3	0.0095 1.0	0.580	+0.007 1%
7	A 30,700 0.24% B 15,000,000 C 15,200,000 101.5%	9,500 25.8% 10,100,000 67.5%	800,000 5.3%	0.300 69.5%	0.008 11.0	0.0008 10.0	0.0074 1.3		-0.043 7.6%
41	A 4,000,000 6.6% B 60,600,000 C 48,000,000 77.3%	2,200,000 56.7% 12,000,000 31.5% 0,600,000 13.6% 1,200,000 3.4% 17,000,000 42.6%	3,050,000 0.03%	0.382 67.1	0.0040 11.4	0.0581 10.2	0.0080 1.4		-0.050 9.8%
21	A 34,350,000 26.2% B 130,000,000 C 125,000,000 91.9%	3,400,000 9.4% 17,000,000 42.6%	3,200,000 2.3%	0.303 63.7	0.0775 13.6	0.0012 10.7	0.0444 7.5		-0.023 4.0%
28	A 1,110,000,000 65.6% B 2,000,000,000 C 1,375,000,000 98.7%	650,000,000 52.6% 305,000,000 20.6%	93,000,000 4.6%	0.351 61.6		0.070 13.3	0.0310 5.0		
35	A 3,875,000,000 154% B 3,200,000,000 C 885,000,000 27.0%		110,000,000 3.4%	0.340 60.8	0.0850 16.0	0.0708 12.4	0.0509 10.0		-0.010 1.7%

A determination of the predominating species of the organisms developing at different temperatures, is seen in Table VIII.

As in Experiment II, there is here shown a marked cutting down of the number of species as the milk ages. It is of interest to note the comparatively large variety of organisms developing at cold storage temperatures on the initial examination. While a number of these species are met with again in plates developing either at 37° C. or at 20° , the *B. formosus*, *B. Ravenel* and *B. cloacæ*, are primarily the types developing at the low temperature. On the final examination the latter organism was in almost pure culture.

A study of the proteid nitrogen in this experiment, carried out as in those previously cited, shows as heretofore, a steady degradation of the casein, decreasing from 75.3 per cent of the total nitrogen to 60.8 per cent. In our other experiments the reduction of casein nitrogen has been much greater. In this connection it may be well to note that in this milk the acid formers were very numerous and the proportion of gelatin liquefiers much lower than is commonly met with in milk stored at low temperatures.

There is, also, in this experiment, a fairly regular increase in the caseose nitrogen as well as in the amino nitrogen, and the difference between the total nitrogen and the sum of the nitrogen estimations is considerably higher than has been noted heretofore leaving, therefore, a larger residue of peptone substances.

The study of the acidity of this sample (Table VII), made as in Experiment III, shows practically no variation until the end of the third week, when it increased to 24 cc. of $\frac{N}{10}$ soda per 100 cc. of milk.

At this time one day in the refrigerator caused a rapid increase in the acidity and a curd on boiling. Two additional days, making a total of three days in the house refrigerator, though there was an acidity of 77.2 cc. per $\frac{N}{10}$ soda per 100 cc. of milk, did not, however, give a spontaneous curd, nor did the milk taste in the least sour. The sample removed from cold storage at the end of the fourth week showed an acidity of 33.6 cc. of $\frac{N}{10}$ soda but there was not at this time the corresponding rapid gain after the one day in the ice box, and after a total of three days when it required 70 cc. of $\frac{N}{10}$ soda, there was no curd even on heating.

TABLE VII. ACIDITY.

Market Milk. Storage Temperature, 0° C.

No. of days in C.S.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.
0	16.8						
7	14						
14	16.8						
21	24	1	34.8 cc. curds on heating	3	77.2 curds on heating		
28	33.6	1	36.8	3	70	8	70.4. No curd until heated
35	44.4	5	solid curd				

TABLE VIII.

Market Milk. Storage Temperature, 0° C.

No. of days.	Incubator.	Refrigerator.	Cold storage.
0	<i>Sarcina lutea</i> . <i>B. siccus</i> (?). <i>M. luteus</i> . <i>Bac. subviscorum</i> . " <i>ferrugineum</i> . <i>Saccharomyces</i> .	<i>Bac. subviscorum</i> (?). <i>B. Ellingtonii</i> .	<i>B. formosus</i> . <i>Bac. rubidium</i> . <i>B. rheni</i> (?). <i>Bac. fulvum</i> . <i>M. lactis</i> . <i>B. aurescens</i> . <i>B. Ellingtonii</i> . <i>B. formosus</i> .
7			
14	<i>M. dissimiles</i> . <i>B. cloacæ</i> .	<i>B. cloacæ</i> . <i>Bac. glaucum</i> . <i>B. Raveneli</i> . <i>B. formosus</i> . <i>B. lividus</i> .	<i>B. formosus</i> . <i>M. citreus</i> . <i>B. Raveneli</i> .
28	<i>B. cloacæ</i> . <i>B. Ellingtonii</i> . <i>M. luteus</i> .	<i>B. formosus</i> . <i>B. cloacæ</i> . <i>B. Raveneli</i> . <i>B. lividus</i> .	<i>B. formosus</i> .
35			<i>B. cloacæ</i> .

One day more gave a curd under these conditions. When it had been kept for five weeks in cold storage and five days after that in the refrigerator a solid curd was formed spontaneously.

Experiment V, Clean Milk, put in Cold Storage November 26, 1906, at a Temperature of 0° C.

This experiment, tabulated in Table IX, is a repetition of No. IV, except that the milk used was clean and not of the market variety. While the increase in the total number of organisms here is comparatively slow it is noteworthy that the count at the end of the fifth week is one of the highest recorded, namely, 7,100,000,000 organisms developing at 0° C. This is a very marked excess over the numbers developing at 20° C. and is observed from the end of the second week to the termination of the experiment. As in the other experiments cited the proportion of organisms developing at 37° C. drops markedly until almost the close of the experimental period when there is, as heretofore, a very marked rise. In this case there is more of a rise than usual, the total number aggregating over 2,000,000,000 organisms per cc.

Though in the beginning of this experiment the liquefying organisms are relatively in very small proportion they increase to such an extent that finally their proportion is unusually high as well as their total number which reaches 535,000,000 organisms per cc.

The predominating species developing at the different temperatures (see Table II) are not markedly different from those cited in the other experiments. As in the others, also, the greater number of species are found on the first examination, this number being gradually cut down for all three temperatures but more pronouncedly for the 0° C. plates. Here we find again the *B. formosus* and *B. solitarius* and an organism not before noted at this temperature—*Mic. aërius*—which, however, is soon crowded out by *B. formosus* and *B. solitarius*.

Table X shows the development of acid in this sample of milk. The acidity, considering the enormous number of organisms, is phenomenally low especially when one considers that on the final examination there were present 1,600,000,000 organisms having an acid reaction on litmus lactose agar. At this time

there was an acidity of but 21.6 cc. of $\frac{N}{10}$ soda for 100 cc. of milk. Neither did the acidity in this experiment increase so greatly when the milk was kept in the house refrigerator. The maximum was reached after the milk had been stored for four weeks and after that kept four days in the ice box, requiring 46.4 cc. of $\frac{N}{10}$ soda per 100 cc. of milk. However, it did not curd on boiling and it kept seven days at ice box temperature before spontaneous curdling took place.

"Blanketed" Milk.

One of the difficulties experienced by dealers in the storage of milk at low temperatures for comparatively long periods is the likelihood of the milk "blanketing," as the trade expresses it. That is, developing into a frothy mass with or without a curd. In this condition it has frequently sufficient force to push up the lid of a forty quart can from which it then escapes as white foam. Such a decomposition may happen to an entire lot of milk or it may affect only a can or two. The source of the milk is no guarantee that this condition will not occur, though it seems to be more frequent in commercially pasteurized than in raw milk. When "blanketing" happens, commercially, the milk is sent to the butter makers.

A large number of samples from various sources, both of dirty and clean milk, were put in cold storage in the hope that some of them would develop this phenomenon but it was several months before such a sample was found. It was then noticed that a glass jar, capped with one of the ordinary pasteboard market caps, was having this cap forced up, so that it was convex instead of concave. A few days later the cap was forced entirely off and raised an inch or so above the top of the jar on a column of very thick, deep yellow cream. There was at this time no visible evidence of frothing. This firm column of thick cream reached a height of an inch and a half before, even with the weight of the cap on the top of it, it fell over. Then the frothy milk covered the top and sides of the jar and gas bubbles could be seen a considerable distance down in the body of the milk itself. This jar was very carefully removed, the column of cream scraped with a sterile spatula into a wide-mouthed

TABLE IX.
Clean Milk. Storage Temperature, 0° C. (Experiment No. V.)

No. of days.	Organisms developing at A 37° C. B 20° C. C 0° C.	Acid formers developing at A 37° C. B 20° C. C 0° C.	Liquefying organisms developing at 20° C.	Casein nitrogen. per cent. 0.451 76.4	Albumin and syn- albin nitrogen. per cent. 0.086 14.5	Casose nitrogen. per cent. 0.057 9.6	Amino nitrogen. per cent.	Total nitrogen.	Difference between total nitrogen and that estimated.
Nov. 26, 1906	A 7,200 159% B 4,516	1,700 27.6% 1,700 57.6%	70 1.5%	0.410 69.4	0.0732 12.4	0.0470 7.9	per cent.	0.590	+ 0.004 0.67%
7	A 3,600 51.4% B 7,000 C 3,100 44.2%	2,000 55.6% 700 22.5%	1,000 14.2%	0.417 70.6	0.0663 11.2	0.0609 10.3	0.0357 6.0	0.0309	- 0.015 2.54%
14	A 865,000 10.7% B 8,050,000 C 8,350,000 103.7%	2,800 0.32% 3,250,000 38.9%	1,310,000 16.2%	0.391 66.2	0.0872 14.7	0.0648 10.9	0.0357 6.0		- 0.012 2.0%
21	A 14,500,000 14.9% B 97,000,000 C 139,000,000 143.2%	38,000,000 59.1%	2,300,000 2.3%	0.343 58.1	0.0945 16.0		0.0297 5.0		
28	A 3,200,000 0.68% B 465,000,000 C 420,000,000 50.5%		54,000,000 11.6%	0.320 54.2	0.0918 16.6	0.1106 18.7	0.0186 3.1		- 0.049 8.36%
35	A 2,730,000,000 68.7% B 3,970,000,000 C 7,100,000,000 178.8%	1,200,000,000 43.9% 1,600,000,000 22.5%	535,000,000 13.4%						

TABLE X. ACIDITY.

Clean Milk. Storage Temperature, 0° C.

No. of days in C. S.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.	Days in house refrigerator.	N NaOH per 100 cc.
	cc.		cc.		cc.		
0	12	2	18.0				
7	16.4	1	16.8	2	19.2		
14	17.2	1	20.4	2	34	3	curd.
21	16.8	1	18.8	2	21.6	3	28.4 cc.
28	21.6	3	25.2	4	46.4	7	solid curd.
35	21.6						

TABLE XI.

Predominating Species of Organisms in Experiment V. Clean Milk.
Storage Temperature, 0° C.

No. of days.	Incubator.	Refrigerator.	Cold storage.
0	<i>Sarcina subflava</i> .	<i>M. aurantiacus</i> . <i>B. acidi lactici</i> (?). <i>Bact. lactis</i> . <i>Streptococcus acidi</i> <i>lactici</i> (?). <i>Penicillium</i>	
7			<i>B. formosus</i> . <i>Mic. aërius</i> .
14	<i>M. citreus</i> . <i>B. Raveneli</i> .		<i>B. solitarius</i> .
28	<i>B. cloacæ</i> . <i>B. ferrugineum</i> . <i>B. formosus</i> .	<i>B. formosus</i> . <i>Sarcina subflava</i> . <i>Saccharomyces</i> .	<i>B. formosus</i> .
35			<i>B. formosus</i> .

sterile flask, the top of the jar wiped off with sterile cotton and the whole removed to the laboratory where it was examined both bacteriologically and chemically. A few days later another sample of milk behaved in the same fashion. These were both market milks and though counts of these particular jars were not originally made others of the same lot were examined and showed from 500,000 to 2,000,000 organisms per cc. It is safe to say, therefore, that these milks were, in all probability, of the usual city type.

The results of the examination of these two samples are given in Tables XII and XIII. It will be noted that the total number of organisms, while fairly high, is not by any means equal to many counts noted in this work where there were no visible evidences of marked deterioration. These milks have been in storage about eight weeks, a considerably longer time than it took other samples to reach a bacterial content of billions per cc.

The acid forming organisms developing at 20° C. were in higher proportion than is commonly seen in milk kept at very low temperatures, and in the second experiment practically all of the colonies developing at 0° C. gave an acid reaction. The liquefying organisms reached a noteworthy proportion.

A study of the predominating species showed comparatively few but most prominent among them were organisms of the coli group. These developed in the incubator and also in the refrigerator. It is to be regretted that for the first sample the set of plates put into cold storage met with an accident which made their study impossible. Corresponding plates in the second experiment gave, not the organisms of the coli group in excess as were expected, but *B. formosus* and *B. pinotus*.

A chemical analysis of the nitrogen distribution showed that the amount of casein had been markedly reduced in both cases and in the second experiment a very unusual quantity of caseose and amino nitrogen had been formed. The acidity of both samples, taken immediately upon their arrival at the laboratory was exceedingly high but in neither case was there any sign of curdling. Vigorous boiling caused a coagulum to form.

A study of the foregoing experiments shows a marked lack of agreement between the digestion of casein and the number of

TABLE XII.
"Blanketed" Milk.

Exp. Number.	Organisms developing at A 37° C. B 20° C. C 0° C.	Acid formers developing at A 37° C. B 20° C. C 0° C.	Liquefying organisms developing at 20° C.	Acidity 1% NaOH per 100 cc.	Casein nitrogen. per cent.	Albumin and syntonin nitrogen. per cent.	Caseose nitrogen. per cent.	Amino nitrogen. per cent.	Total nitrogen. per cent.	Difference between total nitrogen and that estimated.
Sample 1.	A 510,000 6.07% B 8,400,000	280,000 60.9% 4,200,000 50%	1,200,000 14.5%	70.0	0.331 58.4	0.123 21.5	0.075 13.2	0.0055 .97	0.566	0.033 5.83
Sample 2.	A 3,900,000 1.5% B 248,000,000 C 34,133,333 13.7%	400,000 10.2% 120,000,000 48.3%	20,000,000 8.06%	00	0.300 64.9	0.139 19.5	0.107 15.0	0.05 7.04	0.71	0.024 3.58

organisms present. While there is, on the one hand, a progressive loss of casein nitrogen and, on the other, a progressive rise in the total number of organisms, the relations of the two are not constant. For instance, the loss of casein nitrogen during the first week is frequently very great, whereas the rise in the number of organisms during that period is comparatively small, and during the last period when the increase in organisms is much greater than was noted in the preceding weeks the degradation of proteid nitrogen is not correspondingly marked.

TABLE XIII.

"Blanketed Milk." Predominating Species of Organisms.

Experiment.	Incubator.	Refrigerator.	Cold storage.
Sample 1	<i>B. coli</i> group. <i>Mic. luteus</i> . <i>Sterigmatocystis</i> .	<i>B. solitarius</i> Ravenel. <i>B. intestinalis</i> .	
Sample 2	<i>B. subviscorum</i> (?) <i>B. coli</i> . <i>B. coli</i> (var.).	<i>B. cloacæ</i> .	<i>B. formosus</i> . <i>B. pinotus</i> .

The work of Babcock, Russell, Van Slyke, Hart, Vivian and others, on the curing of cheese, would indicate that not bacteria only but enzymes—both those from the cells of the mammary glands and those formed in the milk by the growth of organisms infecting it, are important agents in obtaining hydrolytic nitrogen compounds. Jansen does not find a much greater activity for galactose, the trypsin enzyme of milk, at body heat than at ordinary temperatures. Many others have noted also that the enzymes are much less sensitive to temperature changes below their optimum than they are to changes above that point. It seems probable, then, that the marked chemical alteration in the proteid of milk during storage at low temperatures is caused partly, at least, by the naturally occurring enzymes of the milk rather than by the growth of organisms or enzymes produced by them. If the bacteria play the more important rôle milk kept at room temperature for a comparatively short period should lose casein in proportion to the rise in the number of

organisms. Seven experiments which are given in Table XIV illustrate the changes which were observed.

The samples of milk which have been studied were obtained from four different dairies. A and B were certified milks from mixed herds kept under the best conditions of modern dairying. They were from six to twelve hours old when received at the laboratory. The milk from dairy C was that of one cow, which was caught in the field and milked directly into a clean bottle taken from the laboratory for that purpose. The cow herself was exceedingly clean though the man who milked her left everything in the way of personal cleanliness to be desired. This milk was received at the laboratory for examination within half an hour from the time it was drawn. As will be observed the number of organisms in this sample of milk was very small. Dairy D was of the usual type, perhaps rather better than some which are furnishing city milk. This milk was brought to the laboratory when about six hours old.

All of these samples were examined chemically once in twenty-four hours and plated for bacteriological examination twice daily. The temperature ran from 18 to 22° C.

The increase in organisms in all of these milks was rapid and constant and by the end of the experiment the total number, with two exceptions, was over 100,000,000 per cc.

As one would naturally expect the proportion of acid formers at this temperature was much higher than was found for these organisms in cold storage. The liquefying organisms were represented very well in all of the experiments.

While there is a difference, and a very decided one, in the bacteriological history of milk kept at 18° to 22° as compared with that kept at zero or a little below, there is a much greater difference in the chemical change taking place. Whereas in the course of four or five weeks in storage, at low temperatures, the amount of casein was reduced 50 per cent, we find that at the temperature of the room the milk reaches the souring condition with about 1 per cent only of casein nitrogen being reduced to lower products. There are slight changes in the other proteid constituents but many of them are so small that they are almost within the limits of experimental error.

Unless when milk is stored at low temperatures the chemical

TABLE XIV.
CHANGES IN MILK KEPT AT ROOM TEMPERATURE, 18° to 22° C.
Milk from Dairy A. (Sample No. 52.)

No. of hours.	Total number of organisms. A 37° C. B 15° C.	Acid formers.	Liquefying organisms.	Acidity $\frac{1}{10}$ NaOH per 100 cc. of milk.	Casein nitrogen.	Albumin and syn- tounin nitrogen.	Albumose.	Amido acid.	Total nitrogen.	Sum of nitrogen determined.	Difference between total nitrogen and the sum of that estimated.
					per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.
Oct. 29, 1907, 1.30 p.m.	A 63,000 B 9,800	2,200 6,800	750	16.5	0.483 79.31	0.081 13.30	0.035 5.74	0.035 5.74	0.609	0.624	+0.025 4.10
7½.....	A 9,200 B 11,800	3,800 8,400	1,800	18	0.488 80.13	0.086 14.12	0.021 3.44	0.026 4.26		0.621	+0.012 1.97
19½.....	A 720,000 B 350,000	400,000 210,000	8,000	17.5	0.478 78.48	0.083 13.62	0.032 5.25	0.030 4.92		0.623	+0.014 2.29
26½.....	A 2,300,000 B 1,400,000	900,000 500,000	32,000	17.5	0.477 78.32	0.086 14.12	0.039 6.40	0.026 4.26		0.628	+0.019 3.11
43½.....	A 43,000,000 B 17,100,000	30,000,000 8,200,000	4,200,000	17.5	0.475 77.99	0.070 11.49	0.047 7.71	0.018 2.95		0.610	+0.001 0.16
50½.....	A 100,000,000 B 118,000,000	80,000,000 200,000,000	6,000,000	26	0.473 77.66	0.078 12.80	0.040 6.66	0.008 0.15		0.599	-0.010 1.64

Milk from Dairy A. (Sample No. 39.)

Nov. 6, 1907, 5 hrs. old. Immediate	A B	3,000 3,760	2,000 2,000	200	14.59	0.439 77.01	0.083 14.56	0.032 5.61	0.027 4.73	0.570	0.581	+0.011 1.93
11 hrs. old.....	A B	3,450 5,000	1,700 2,000	1,220								
28 hrs. old.....	A B	6,170,000 8,000,000	5,500,000 3,000,000	1,787,500	10	0.437 76.66	0.077 19.50	0.036 6.31	0.028 4.91		0.578	+0.008 1.40
36 hrs. old.....	A B	6,000,000 9,200,000	4,200,000 7,400,000	2,000,000								
52 hrs. old.....	A B	72,500,000 148,000,000	48,000,000 90,000,000	20,000,000	71.85	0.439 77	0.054 9.47	0.062 10.37	0.023 4.03		0.578	+0.008 1.40

Milk from Dairy A. (Sample No. 53.)

Nov. 11, 1907, 10 a.m., 6 hrs. old Immediate	A B	3,500 2,900	1,920 1,800	150	13.5	0.453 77.83	0.081 19.91	0.028 4.81	0.029 4.98	0.582	0.591	+0.009
Nov. 11, 4:30 p.m.	A B	3,050 3,050	1,290 2,000	200	14.0							
Nov. 12, 9 a.m....	A B	172,000 192,000	128,000 100,000	42,000	15	0.463 79.56	0.067 11.51	0.039 6.70	0.015 2.56		0.584	+0.002
Nov. 12, 4:30 p.m.	A B	780,000 740,000	500,000 185,000	220,000	15.5							
Nov. 13, 9 a.m....	A B	51,000,000 51,750,000	41,000,000 35,350,000	400,000	21.5		0.022 3.78	0.044 7.56	0.019 3.26			
Nov. 13, 3 p.m. ..	A B	89,000,000 67,000,000	82,000,000 42,000,000	2,000,000	22.5	0.450 77.31	0.076 19.06	0.041 7.04	0.024 4.13		0.501	+0.009

TABLE XIV—Continued.
Milk from Dairy A. (Sample No. 56.)

No. of hours.	Total number of organisms. A 37° C. B 15° C.	Acid formers.	Liquefying organisms.	Acidity per 100 cc. of milk.	Casein. per cent.	Albumin and syn- tonin nitrogen. per cent.	Albumose. per cent.	Amido acid. per cent.	Total nitrogen. per cent.	Sum of nitrogen determined. per cent.	Difference between total nitrogen and the sum of that estimated. per cent.
1907											
Nov. 14, 1 p.m. . .	A 2,120 B 1,040	900 400	100	14	0.464 78.37	0.078 13.17	0.047 7.93	0.031 5.23	0.592	0.620	+0.028 4.72
Nov. 15, 9 a.m. . .	A 58,000 B 87,500	51,500 22,200	19,500	14.5	0.459 77.53	0.077 13.00	0.044 7.43	0.024 4.06	0.592	0.606	+0.014 2.36
Nov. 16, 9 a.m. . .	A 20,200,000 B 15,000,000	11,000,000 8,000,000	19,000,000	19.0	0.452 76.35	0.085 14.35	0.038 6.41	0.016 2.70	0.592	0.591	-0.001 0.16

Milk from Dairy B. (Sample No. 63.)

Nov. 25, 9 a.m. . .	A 1,525 B 1,050	825 700	120	12.5	0.437 77.76	0.065 11.56	0.040 7.11	0.020 3.55	0.562	0.562	
Nov. 25, 4 p.m. . .	A 2,250 B 1,200	1,350 800	100	12.5							
Nov. 26, 9 a.m. . .	A 200,000 B 840,000	146,000 400,000	17,000	13.0	0.430 76.64	0.081 14.43	0.028 4.99	0.018 3.20	0.561	0.557	-0.004
Nov. 26, 4 p.m. . .	A 2,520,000 B 3,100,000	2,160,000 1,320,000	1,000,000	16.0							
Nov. 27, 9 a.m. . .	A 187,000,000 B 186,000,000	138,000,000 138,000,000	10,000,000	22.0	0.420 76.22	0.080 14.51	0.029 5.36	0.023 4.17	0.551	0.552	+0.001

Milk from Dairy C. (Sample No. 61.)

Nov. 19, 12 m. . . .	A B	1,000 1,000	900	200	9.0	0.407 76.57	0.077 14.25	0.052 9.62	0.009 1.66	0.510	0.515	+0.005 0.92
Nov. 20, 9 a.m. . . .	A B	17,000 20,000	16,500 10,000	1,000	11.5	0.396 72.39	0.004 17.18	0.037 6.76	0.033 6.03	0.547	0.560	+0.013 2.59
Nov. 21, 9 a.m. . . .	A B	700,000 120,000	60,000 662,000	140,000	11.0	0.395 73.14	0.095 17.59	0.034 6.29	0.023 4.25	0.540	0.547	+0.007 1.29
Nov. 22, 9 a.m. . . .	A B	100,000,000 6,200,000	65,000,000 5,000,000	215,000	14.0	0.382 70.74	0.088 16.29	0.053 9.81	0.012 2.22	*0.540	0.535	-0.005 0.92
Nov. 22, 4 p.m. . . .	A B	120,000,000 224,000,000	85,000,000 134,000,000	500,000	12.0	0.384 71.77	0.104 20.18	0.048 8.97	0.006 1.12	0.535	0.546	+0.011 2.03

* This calculation is made on the basis of the mean of all the determinations.

Milk from Dairy D. (Sample No. 64.)

Nov. 25, 9 a.m. . . .	A B	16,800 48,000	6,400 33,000	4,000	13.5	0.374 74.94	0.045 9.01	0.037 7.41	0.024 4.80	0.409	0.480	-0.019
Nov. 25, 4 p.m. . .	A B	314,000 650,000	254,000 440,000	270,000	13.5							
Nov. 26, 9 a.m. . .	A B	6,000,000 12,000,000	3,700,000 4,600,000	1,800,000	20.0	0.363 73.63	0.068 13.79	0.020 5.88	0.019 3.85	0.403	0.479	-0.014
Nov. 26, 4 p.m. . .	A B	120,000,000 120,000,000	106,000,000 110,000,000	1,000,000	73.0 (sour)							

behavior of the organisms inhabiting it is totally changed it seems likely that the digestion which is observed there is due primarily to enzymes of animal origin rather than to the growth of bacterial flora.

The production of so large a quantity of acid as is formed on keeping milk for a considerable length of time in cold storage makes it possible that a certain amount of chemical action may go on between the milk itself and the metal of the container, which action might result in aiding the decomposition of the proteid. As has been stated previously, the milk reported in the foregoing experiments was stored in tin cans.

To determine the relative keeping quality of milk in glass the following analyses, both bacteriologically and chemically, were made of milk which had been in cold storage for periods varying for from ten days to twenty months. These jars were paper capped and therefore quite free from contact with any metal. The results of their examination are given in Table XV. It will be observed that a very decided decrease in casein nitrogen has taken place, and in the old samples, where the analysis has been extended to the proteolytic decomposition products, they have increased very decidedly.

It is proposed, in the pursuance of this study, to determine more fully the comparative keeping qualities of milk in metal and glass containers. The results here offered are of interest, however, especially when one considers the length of time the milks were stored, the high acidity and the comparatively good condition of the milk so far as odor, taste and appearance is concerned.

SUMMARY.

Bacteria in milk increase in numbers when the temperature is maintained at or a little below 0° C. This temperature is below that ordinarily assigned as the limit of bacterial multiplication.

Milk has been kept for periods ranging from a few days to almost two years at a temperature of 29° to 31° F., and also at 32° F. It has been kept in packages of ten quarts and one quart. It has been the cleanest milk obtainable, by the most carefully enforced refinements of modern dairying; and it has also been

TABLE XV.

Description of sample.	Total number of organisms at 20° C.	Acid forming organisms.	Liquefying organisms.	Total nitrogen	Casein nitrogen.	Albumin and syntonin nitrogen.	Caseose nitrogen.	Amido acid nitrogen.	Difference between total nitrogen and the sum of that estimated.	Acidity $\frac{1}{10}$ N NaOH per 100 cc. of milk.
(1) Clean milk; quart jar. In cold storage from Feb. 19, 1906, until Sept. 12, 1906. Did not freeze. Did not curd on heating, but did curd in a few hours at room temperature.	250,000,000	60,000,000	70,000	per cent. 0.705	per cent. 0.470					
(2) Clean milk; quart bottle. In cold storage from Sept. 16, 1906, until Sept. 20, 1906.	60,000	10,000	20,000	0.00	0.474					
(3) Clean milk; quart bottle. In cold storage from Sept. 16, 1906, until Sept. 26, 1906.	20,000	2,000	5,000	0.00	0.415					
(4) Market milk; qt. bottle. In cold storage from Sept. 16, 1906, until Sept. 20, 1906.	4,450,000		505,000	0.50	0.452					
(5) Market milk; qt. bottle. In cold storage from Sept. 16, 1906, until Oct. 1, 1906.	148,500,000	88,000	460,000	0.03	0.447					
(6) Clean milk; quart bottle. In cold storage from Feb. 19, 1906, until Sept. 25, 1907. Very fine slight curd on boiling. Did not curd at room temperature until the fifth day.	A 32,000,000 B 97,000,000 C 920,000	9,200,000 300,000	14,200,000	0.548	0.321 58.69	0.076 13.86	0.0915 16.69	0.046 8.59	-0.0135 2.40	79.5
(7) Clean milk; quart bottle. In cold storage at 32° to 20° F. from Feb. 19, 1906, until Oct. 21, 1907. Curded on sixth day.	A 10,000,000 B 18,000,000	3,000,000 2,000,000	14,200,000	0.583	0.292 60.08	0.064 10.37	0.145 24.87	0.072 13.34	-0.010 1.71	101
(8) Clean milk; quart bottle. Same history as sample No. 7	A 9,000,000 B 3,000,000	1,000,000	1,000,000	0.572	0.208 62.09	0.076 13.23	0.118 20.62	0.064 11.18	-0.010 2.79	100

market milk produced in the ordinary dirty stable and subjected in transit to the usual careless handling.

Bacterial growth at the end of a week, even in the cleanest milk which contained as low as 300 organisms to the cc., was pronounced. There was a steady increase in the number of organisms for five or six weeks, and at their maximum they numbered hundreds of millions. Occasionally they passed the billion mark per cc.

Continued exposure to a temperature of 29° to 31° F. causes, after a lapse of from seven to twenty-one days, the formation of small ice crystals which gradually increase until the milk is filled with them and there may be an adherent layer on the walls of the vessels. The milk does not freeze solidly. In spite of the fact that the milk was a semi-solid mass of ice crystals, the enormous increase in bacteria which this study shows, took place. Though the bacterial content was numerically in the hundreds of millions per cc. there was neither odor nor taste to indicate that such was the case. Neither did the milk curd even on heating, and it was not until the bacterial content began to fall, and organisms of putrefaction were under way, that the use of the milk for household purposes would, to the ordinary observer, become contra-indicated.

A classification on a chemical basis of the organisms occurring at these low temperatures, shows that there were constantly present bacteria which formed acid and bacteria which acted upon proteid. There were also neutral organisms, which formed neither acid nor alkali and did not act upon gelatine. The acid forming organisms were generally in relatively smaller numbers than are found when milk is kept at higher temperatures, and the liquefying organisms were more numerous. Certain species, such as *B. formosus*, *R. solitarius*, and *B. Ravenel*, were especially resistant to cold and frequently were the predominating species, or almost in pure culture at the close of the experiment.

A very marked difference in both the number and kind of organisms which developed on the plates was noticed, depending upon the temperature at which the plate was incubated. In certain experiments the maximum number grew at 37° C. In others the temperature at which the milk was stored served best for colony formation. The relative number of organisms growing

at 37° C., 20° C., and 0° C., or a little below, varied greatly also with the length of time that the milk had been kept in storage, the organisms developing at body temperature being ordinarily greatly in excess at the beginning of the experiment and diminishing until near its close, when a sharp rise was apt to take place.

The determination of the acidity showed that after a few weeks a much higher acid content was reached than is ordinarily required for the spontaneous separation of curd, which, however, seldom happened. Milk having this high acidity, when placed in an ordinary ice chest, increased in acid content but did not curd for days after exposure to the higher temperature.

The chemical study of the proteid of milk in cold storage, showed that the casein was rapidly digested until finally more than 50 per cent of it was changed to soluble compounds. Caseoses, amido acids, and probably peptones, increase apparently at the expense of the digested casein. The rapidity with which this digestion takes place varies in different samples, but at the expiration of two weeks it is pronounced.

What the effect of the low temperatures is on the carbohydrate constituents of the milk remains for further study. That an interesting decomposition, and one which varies from that occurring at higher temperatures, takes place is indicated by the very high acid content of the milk noted throughout this investigation.

Similar studies, conducted on samples of very fresh milk kept at the temperature of the laboratory (about 18° to 22° C.), shows a very decided difference chemically from the decomposition of milk in cold storage. Bacterial growth at room temperature is, of course, rapid and profuse. The acid forming organisms are, as has been found by other observers, in high proportion and the liquefying organisms are relatively lower. The chemical change observed is, by comparison with that occurring in cold storage, almost nothing. At the curding point only about 1 per cent of the casein has been changed to soluble products, and spontaneous curding is observed, ordinarily, when the acid content falls between 23 and 28 cc. $\frac{N}{100}$ sodium hydrate per 100 cc.

I am greatly indebted to Dr. St. John and Mr. Hepburn, both of this laboratory, for their assistance in the bacteriological and chemical work involved in this investigation.

FURTHER EXPERIMENTS ON THE MECHANISM OF SALT GLYCOSURIA.

BY FRANK P. UNDERHILL AND ISRAEL S. KLEINER.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

(Received for publication, February 15, 1908.)

In a previous paper upon the mechanism of salt glycosuria Underhill and Closson¹ concluded that "when sodium chloride is injected into the venous circulation of the rabbit, polyuria and glycosuria are in evidence, probably as a result of an increased permeability of the kidney. The permeability of the kidney may be decreased by injection of a mixture of sodium chloride and calcium chloride, as is indicated by the temporary decreased flow of urine and diminished or inhibited excretion of sugar. Further evidence that this form of glycosuria is of renal origin is furnished by the observation that during the appearance of sugar in the urine hypoglycæmia is noted, whereas the sugar content of the blood becomes normal, or hyperglycæmia obtains, when the excretion of sugar in the urine is inhibited by a mixture of sodium chloride and calcium chloride. Injection of sodium chloride into the cerebral arterial circulation induces glycosuria with no polyuria, but with an accompanying hyperglycæmia. The increased content of sugar in the blood may be referred to disturbances of respiratory processes, dyspnœa, provoked by the introduction of sodium chloride."

A more recent statement of McGuigan and Brooks² that "the permeability of the kidney is of small import in the mechanism of experimental glycosuria," was based upon the following experiments.

¹ Underhill and Closson: *Amer. Jour. of Physiol.*, xv, p. 321, 1906. References to the older literature are given here.

² McGuigan and Brooks: *Amer. Jour. of Physiol.*, xviii, p. 256. 1907.

EXPERIMENT I. Dog. Female. Weight, 4.5 kilos. Ether anæsthesia. In twenty minutes 40 cc. $\frac{M}{4}$ CaCl_2 was run into the jugular vein. A decrease in the flow of urine followed. A sugar solution (10 cc. 2 per cent dextrose) was injected into the renal artery. The urine was flowing at the rate of 1 cc. every five minutes and did not contain sugar. After injection of the sugar solution, when 1 cc. of urine had run from the ureters, sugar was easily demonstrable in it.

EXPERIMENT 2. Rabbit. Weight, 2100 grams. Urethane anæsthesia.

Time.	Salt injected.		Urine.	Remarks.
	cc.		cc.	
10:45 } 11:12 } 11:15 }	45	0.9 per cent NaCl	6.0	No sugar.
11:28 } 11:40 } 11:48 }	25 } 20 } 21 }	40 cc. $\frac{M}{4}$ CaCl_2 to 260 cc. 0.9 per cent NaCl	5.0 14.0 10.0	No sugar. " "
12:00 } 12:20 } 12:28 }	20 } 40 } 10 }		13.5 40.0 9.0	" " "
12:29	1	1 per cent dextrose in 0.9 per cent NaCl		
12:32 } 12:38 }			5.0	Sugar. "
12:40 } 12:57 }	2 } 15 }	40 cc. $\frac{M}{4}$ CaCl_2 to 260 cc.	4.0 2.0	Sugar, large amount. " "
1:20	25	0.9 per cent NaCl	23.0	"
1:34 } 1:39 }	3 } 12 }	$\frac{M}{4}$ CaCl_2	3.0 5.0	Trace sugar.
1:44 } 2:04 }	10 } 20 }	"	1.0 7.0	No sugar.
2:12	50	"	10.0	(Squeezed from bladder—no sugar).
2:15	10	1 per cent dextrose in 0.9 per cent NaCl		
2:16				Sugar in the urine in large quantity.

The method of procedure employed in Experiment 1 shows at once that McGuigan and Brooks must have had a misconception of the conclusions drawn by Underhill and Closson. In stating that "the permeability of the kidney may be decreased by injection of a mixture of sodium chloride and calcium chloride, etc.," we did not intend to imply that this refers to the *normal kidney*.

Indeed a careful study of our paper would show such a conclusion unwarranted since no experiments were made with the normal kidney. And as our experiments were carried out with rabbits we can not extend our conclusions to the kidney of the dog. Our intention was to point out that an increased permeability induced by sodium chloride could be decreased to the point where sugar is not allowed to pass. The first experiment of McGuigan and Brooks, dealing as it does with the dog under conditions where the renal permeability has not previously been altered, fails to controvert our original conclusion.

The protocol¹ of an experiment² in repetition of Experiment 2 of McGuigan and Brooks follows.

EXPERIMENT A. Female rabbit. Weight, 1600 grams. No anæsthesia. The injections were made gradually into the marginal ear vein between the periods of time indicated. The volume of urine excreted was not recorded.

Time.	Solution injected.	Remarks.
	cc.	
2:43 } 3:12 }	45 0.9 per cent NaCl.	
3:42 } 3:59 } 4:07 }	46 } 47 }	No sugar in urine.
4:17 } 4:25 }	45 } 7 }	Sugar present. Respiration rapid.
4:25 } 4:41 }	40 cc. $\frac{N}{4}$ CaCl_2 to 260 cc.	Sugar present. Respiration slow and shallow.
4:41 } 5:04 }	16 } 24 }	Sugar present. Respiration shallow. Lips and ears blue.
5:13 } 5:25 } 5:30 }	18 } 27 }	Respiration deeper. Tremors.
	$\frac{N}{4}$ CaCl_2	Respiration very poor.
		Animal killed and blood collected. Sugar content of blood = 0.25 per cent. Urine in bladder contained sugar.

The results of this experiment, which is but one of many, demonstrate beyond question that calcium chloride in the

¹ The methods employed throughout were those indicated in our former investigation.

² In one experiment a rabbit weighing 1860 grams excreted sugar in the urine after the injection in 14 minutes of 18 cc. of the calcium chloride—sodium chloride solution (40 cc. $\frac{N}{4}$ CaCl_2 to 260 cc. 0.9 per cent NaCl).

strength employed by McGuigan and Brooks is sufficient alone to call forth glycosuria. Examination of the blood proved that when calcium chloride produces glycosuria hyperglycæmia is present. In their attempt to imitate our conditions McGuigan and Brooks injected 45 cc. of 0.9 per cent sodium chloride solution presumably in order to increase the permeability of the kidney whereas many times that quantity will give but little evidence of increased permeability so far as sugar is concerned. On the other hand the strength of calcium chloride employed by them was so great that it was capable of producing glycosuria of itself through action upon the respiratory mechanism as our experiment given above shows. The results of these authors are in reality in harmony with our own. They state that "in all the cases examined by us we found an increase in the sugar content of the blood." In other words, they induced hyperglycæmia with calcium chloride, not hypoglycæmia which results when glycosuria occurs after injection of sodium chloride into the venous circulation. Furthermore in their protocol, Experiment 2, at 12:29 they injected 1 cc. 1 per cent dextrose in 0.9 per cent sodium chloride solution, at 12:32 they found sugar in the urine and again at 12:38. At 12:40 and 12:57 sugar was present "*in large amount.*" *What was its source? It scarcely could come from the ten milligrams injected. Our explanation is that the sugar found in the urine owes its appearance to the content of calcium chloride injected, and our experimental observations are in accord with this.*

We have undertaken Experiment B to determine whether after glycosuria had been induced by sodium chloride and inhibited with calcium chloride dextrose could be introduced intravenously without reappearing in the urine. The outcome of such an experiment would obviously settle the question whether calcium chloride really does prevent sugar from passing the kidney. This was the point aimed at by McGuigan and Brooks but which their experiments failed to elucidate.

EXPERIMENT B. Female rabbit. Weight, 1900 grams. No anæsthesia. The solutions were injected into the marginal ear vein between the periods indicated. The volume of urine excreted was not recorded.

Time.	Solution injected.		Remarks.
	cc.		
8:44			No urine in bladder.
8:50	41	0.9 per cent NaCl	
8:59	39		
9:11	41		
9:20	13		
9:27	27		
9:32	41		
9:37	42		No sugar in urine.
9:43	41		
9:52	42		Trace of sugar in urine.
10:01	41		
10:21	42		" " " "
10:33	43		Sugar present " "
10:53	90		" " " "
11:02	46	975 cc. 0.9 per cent cent NaCl + 25 cc. $3\frac{3}{5}$ CaCl ₂	
11:13	43		
11:19	45		Sugar present in urine.
11:28	45		Trace of sugar " "
11:47	49		No sugar " "
11:57	45		" " " "
12:22	93		" " " "
12:30	2	{ 1 per cent dextrose 0.9 per cent NaCl	
12:33			" " " "
12:54	3	ditto	
12:55			" " " "
1:06	5	ditto	
1:08			" " " "
1:18	10	ditto	
1:22			" " " "
1:29	10	ditto	
1:34			" " " "
1:39			" " " "

Under the experimental conditions outlined sugar may be injected into the blood without appearing in the urine, in other words, calcium chloride will prevent sugar appearing in the urine even though *free* sugar is introduced into the blood. In this connection may be cited the behavior of dextrose when injected

into normal rabbits. According to Blumenthal¹ quantities of dextrose up to 2.5 grams may be intravenously introduced into normal rabbits within five minutes without the reappearance of sugar in the urine. These observations upon rabbits have been corroborated by Comessatti.² Some of the data of the latter are here reproduced.

Weight of rabbit.	Quantity of dextrose intravenously injected without subsequent glycosuria.
<i>kilos.</i>	<i>gms.</i>
2.50	2.10
3.10	2.10
3.15	2.40
2.65	2.00
2.10	1.50
2.20	1.50
2.10	1.50
2.00	1.50

From these data it is readily seen that the *normal* kidney possesses a marked resistance to the passage of free sugar introduced into the blood. Yet according to McGuigan and Brooks "the real essential for glycosuria, however, is the presence of uncombined sugar in the blood. When this is present even in minute quantities, sugar passes into the urine." If this statement is correct why is it that as much as 2.5 grams of dextrose (250 times the quantity employed by McGuigan and Brooks) can be injected into the blood of a normal rabbit without glycosuria as shown by Blumenthal and by Comessatti? It is our opinion that the conditions necessary for glycosuria may be twofold, (a) an abnormally high sugar content of the blood, or (b) an increased permeability of the kidney, with consequent hypoglycæmia.

CONCLUSIONS.

Renewed investigation has afforded no occasion to modify the conclusions already reached regarding the mechanism of salt glycosuria.

¹ Blumenthal: *Beiträge zur chemischen Physiologie und Pathologie*, vi, p. 329, 1905.

² Comessatti: *Beiträge zur chemischen Physiologie und Pathologie*, ix, p. 67, 1907.

Under appropriate conditions in the rabbit glycosuria due to renal permeability induced by sodium chloride injections can be inhibited by injections of calcium chloride.

If calcium chloride is appropriately introduced glycosuria fails to be evinced even when free sugar is injected in addition.

NOTE ON THE INFLUENCE OF MEAT ON THE DIMETHYL-AMIDOBENZALDEHYDE (EHRlich'S ALDEHYDE) REACTION OF THE URINE.

By C. A. HERTER.

(Received for publication, January 28, 1908.)

Various observers have noticed that the cherry-red coloration of the urine induced by an acid solution of Ehrlich's aldehyde is apt to be particularly strong under some pathological conditions and personal experience has led me to consider these pronounced examples of the reaction especially frequent in affections of the digestive tract. A satisfying explanation of the chemical basis of the reaction is still wanting and the difficulty in supplying it is due in part to the circumstance that this highly reactive aldehyde forms cherry-red compounds in the urine with more than one substance. Thus Bauer has brought forward evidence that the reaction depends on the urobilinogen¹ of the urine and I have shown that the reaction is at least augmented by the administration of skatol. I now have evidence that indolacetic acid is one of the urinary constituents that may react with the aldehyde, although not strongly except in concentrated urines. It is not improbable that there are other ways in which a typical dimethylamidobenzaldehyde color reaction may be obtained in the urine. Until all the possible origins of the reaction are known, no satisfactory general statement can be made in respect to any pathological significance which the exaggerated reaction may at times possess. Meanwhile it seems worth while to mention the important influence of a meat diet upon the Ehrlich reaction, for unless this be realized there is a likelihood of falling into the error of ascribing a pathological meaning to a phenomenon which is at times in reality physiological in nature. As patients with

¹ It is probably this fact that has led Fr. Müller to regard the reaction as connected with hepatic disease. I am told that in Müller's clinic the strongest reactions have been noted in diseases of the liver, in which the aldehyde reaction is frequently obtainable in the cold.

disorders of intestinal digestion are often given a diet containing considerable meat, it is easy to see how this fact may complicate the interpretation of a strong aldehyde reaction.

Attention was first attracted to the influence of meat by the fact that a meat-fed dog whose urine gave an intense cherry-red reaction with Ehrlich aldehyde promptly secreted urine which failed to give this reaction when the diet was altered by substituting milk for meat. On returning to a meat diet the urine regained its capacity to react typically with paradimethylamido-benzaldehyde. This observation was repeated with similar results on several dogs. Several trials were then made on men and these trials indicated that an abundance of beef caused an intensification of the aldehyde reaction, whereas a restriction in meat was followed by a distinct decline in the intensity of the reaction, although not necessarily by its abolition.

With a view to finding out what constituent of meat food is concerned with the effect on the urine, three sets of experiments were made. In one of these the milk which served as food was mixed with dog's blood, in order to determine whether the hæmoglobin might be so transformed as to exert an effect on the reaction. No positive effects were obtained from the quantities of blood that were used. In other experiments Liebig's extract of beef was added to the milk. Here there was noted a moderate rise in the intensity of the aldehyde reaction, although the extract itself has no constituent that gives the reaction. These observations were made on dogs. Finally, a healthy man, whose urine habitually gave a strong Ehrlich aldehyde reaction while he was taking a meat diet, was fed ground beef from which nearly all soluble pigments had been washed out by a stream of water. In this case, before beginning the experiment of feeding the colorless meat, the urine was rendered almost irresponsive to Ehrlich's aldehyde by a diet of milk, eggs and bread for a week. On taking one kilo of washed beef in two successive meals, no increase in the reaction was observed, this result contrasting with similar experiments made with unwashed beef in the same quantity. The colorless meat of fish gave results like those obtained with washed beef.

It may be stated that the free use of beef by three healthy men was followed regularly by an intensification of the Ehrlich alde-

hyde reaction of the urine. This intensification is dependent on the presence of the coloring matter of the meat. I hope soon to be able to offer an explanation of this influence of the coloring-matter contained in meat.

It should be mentioned that in the experiments referred to in this note the urines were regularly diluted to a specific gravity of 1010 before testing them. Unless this precaution be taken, there is a liability to error in making comparisons of the intensities of the reactions.

PROCEEDINGS OF THE
AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS,

IN SESSION AT THE

University of Chicago, December 30, 1907, to January 2, 1908.

EDITED BY THE SECRETARY.

Summary of Meetings.

1. *Monday afternoon, December 30.*
2. *Tuesday morning, December 31.* Joint session with the American Physiological Society.
3. *Wednesday morning, January 1.* Joint session with the Biological Section of the American Chemical Society in affiliation with Section C (Chemistry) of the American Association for the Advancement of Science.
4. *Thursday morning, January 2.*

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

University of Chicago, December 30, 1907, to January 2, 1908.

First Meeting.

Physiology Building. Monday afternoon, December 30.

Presiding officer: The President, Russell H. Chittenden.

THE RATE OF OXIDATION OF THE SUGARS IN AN ACID MEDIUM.

By H. H. BUNZEL (by invitation).

*(From the Laboratory of Biochemistry and Pharmacology, University of
Chicago.)*

The rate of oxidation of the most common sugars was studied, using copper acetate as the oxidizing agent in an acetic acid medium of known concentration. The sugars could be arranged according to their rate of oxidation in the order: Levulose (oxidizes fastest), galactose, mannose, glucose, maltose and lactose (oxidizes slowest). No satisfactory velocity constants could be obtained. The order of the reaction was determined by van't Hoff's method and was found to be 5. Acidity plays a very important rôle in the speed of oxidation, by decreasing the number of oxygen ions. The velocity was determined with different concentrations of acid.

To determine the relative rate of oxidation of the sugars, the velocities at the start were compared. The figures obtained were:

Lactose.....	1	Galactose.....	8.72
Maltose.....	1.15	Mannose.....	8.72
Glucose.....	5.71	Levulose.....	55.13

ON THE EFFICIENCY OF THYMOL AND REFRIGERATION FOR THE PRESERVATION OF URINE AS SHOWN BY COMPARATIVE ANALYSES FOR THE VARIOUS NITROGENOUS CONSTITUENTS AT THE END OF 24, 48, 72 AND 96 HOURS.

BY P. B. HAWK AND H. S. GRINDLEY.

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois.)

The following series of tests were made to determine the variations occurring in the percentage content of the nitrogenous constituents of the urine when thymol and refrigeration were adopted as means of preservation:

(I) The analysis of three individual 24-hour samples of urine at the end of 24, 48 and 96 hours.

(II) The analysis of four 48-hour composite samples of urine at the end of 48, 72 and 96 hours.

Comparative analyses were made on portions of each of these urines after dilution with two volumes of water.

(III) The analysis of twenty-two individual 24-hour specimens of urine at the end of 24 and 96 hours.

In each instance the 24-hour urine was collected in a bottle which had been thoroughly cleaned and subsequently rinsed with a 10 per cent alcoholic solution of thymol. After carefully drying the interior of the bottle, about 0.2 gram of powdered thymol was added. While the 24-hour urine was being collected the bottles were kept in a refrigerator (temp. 13° to 20° C.) and transferred to cold storage (temp. 7° to 10° C.) immediately after the sample was completed and portions removed for analysis.

The urine samples, with the exception of those included in Series III, were subjected to the following analyses: Ammonia, urea, uric acid, creatinin and total nitrogen. The urines of Series III were analyzed for ammonia and uric acid. The methods of analysis were those proposed by Folin in each case except for total nitrogen, in which instance the Kjeldahl method was employed. The data regarding the preservation of the urine for a period of 96 hours may be summarized in tabular form as follows:

Determination.	Average percentage content at the end of 96 hours, the content at the end of 24 hours being taken as 100 per cent.	
	24 hours.	96 hours.
Ammonia.....	100	102.8
Creatinin.....	100	100.15
Uric acid.....	100	100.6
Urea.....	100	96.4
Total nitrogen	100	100.1

COMPARATIVE TESTS OF SPIRO'S AND FOLIN'S METHODS FOR THE DETERMINATION OF AMMONIA AND UREA.

By PAUL E. HOWE AND P. B. HAWK.

(From the Laboratory of Physiological Chemistry, Department of Animal Husbandry, University of Illinois.)

Spiro's combination method for the determination of ammonia and urea in the same specimen of urine appeared in a recent issue of Hofmeister's *Beiträge* (ix, p. 481, 1907). It embraces essentially the Folin technique for the determination of ammonia coupled with the Mörner-Sjöquist principle for the estimation of urea.

Check tests made by Spiro's and by Folin's methods upon solutions of urea and of ammonium chloride-urea gave practically theoretical results for both urea and ammonia. When urine was analyzed, however, the Spiro method gave *higher figures in every instance* for both ammonia and urea than did the method of Folin. The average data from a large number of tests on check solutions are given below in tabular form:

Solution examined.	Ammonia. Per cent recovered.		Urea. Per cent recovered.	
	Spiro.	Folin.	Spiro.	Folin.
NH ₄ Cl urea.....	99.38	99.46	99.98	99.46
Urea	100.02	99.17

A few typical results on the urine follow:

No. of urine sample.	Ammonia, grams.		Urea, grams.	
	Spiro.	Folin.	Spiro.	Folin.
I	0.712	0.697	16.278	15.311
II	0.699	0.696	16.801	15.203
III	0.673	0.665	18.688	16.596

Check tests in which solutions of pure creatinin, hippuric acid, and uric acid were analyzed indicated that all the creatinin and

hippuric acid were determined in Spiro's method for urea, thus accounting for the excess nitrogen above that determined by the Folin method.

A STUDY OF THE RELATIVE THERAPEUTIC VALUE OF ANTITOXIC GLOBULIN SOLUTION AND THE WHOLE SERUM.

By EDWIN J. BANZHAF (by invitation.)

(From the Research Laboratory of the Department of Health, New York City.)

Cruveilhier, in a paper in the *Annales de l'Institut Pasteur* (xviii, p. 249) quotes Roux, Marfan, Martin and Momont, as finding that the dose of antidiphtheric sera most efficacious is not always the one which contains the greatest number of antitoxic units. They assert that in the serum, there are, besides antitoxin, other protective substances, whose therapeutic value is ignored in the present measurement of antitoxic units.

Cruveilhier to test this point carried out a series of experiments with guinea pigs infected with diphtheria bacilli. He compared sera of different antitoxic strength from several horses, as to their value preventively and curatively. In his *preventive* experiments, he injected, subcutaneously, a quantity of serum proportional to the weight of the animal and, 24 hours later, inoculated subcutaneously a fatal dose of diphtheria culture. In his *curative* experiments, he injected the guinea pigs, subcutaneously, with a diphtheria culture; and divided the animals into two lots. From the second to the sixteenth hour after inoculation, each animal of one lot received subcutaneously 0.1 cc. of the serum of the lesser unit content; the second lot 0.1 cc. of the serum of the greater unit content. In all of his preventive and curative experiments, the sera of the lesser unit content were more efficacious than those of the greater.

His results apparently showed that it was the quantity of serum, rather than the number of antitoxic units, which was of therapeutic value.

In a recent report "On the relative value of antitoxin and other curative substances in antidiphtheric serum," that appeared in the November number of the *Proceedings of the Society for Experi-*

mental Biology and Medicine, Steinhardt and Banzhaf reported results, which were diametrically opposed to those of Cruveilhier and the authors he cites.

For further study we have deemed it interesting and important to compare the antitoxic globulin solution (Gibson), and the antitoxic globulin fractions (Banzhaf and Gibson), with the whole antitoxic plasma. Infecting three series of guinea pigs with different strains of the diphtheria bacillus, we have compared the following.

A mixture of citrated plasma of 700 units (before eliminating the non-antitoxic proteins and concentrating) with the finished product of antitoxic globulin solution of 1650 units; a mixture of citrated plasma of 450 units (before eliminating the non-antitoxic proteins and concentrating) with the finished product of 1275 units, and with the three fractional precipitations of the citrated plasma (obtained by precipitating the antitoxic globulins from the plasma at concentrations corresponding to 3.3 cc., 3.3 to 3.8 cc. and 3.8 to 5.0 cc. of saturated ammonium sulphate in 10 cc.). The first fraction of the antitoxic globulin solution contained 1025 units per cc.; second fraction, 1175 units, and the third, 1550 units.

From the results obtained in the comparison of the citrated plasma, with the antitoxic globulin solutions and their fractions, it is obvious that the preventive and curative agent, the antitoxic unit, is in no way impaired in the process of eliminating the albumins and other non-antitoxic proteins by the salting out methods employed, and the final dialyzation of the concentrated product.

THE QUANTITATIVE CHANGES DURING IMMUNIZATION IN THE BLOOD OF HORSES AND THE RELATION OF THE SERUM GLOBULIN TO DIPHTHERIA AND TETANUS ANTITOXIN CONTENT.

By EDWIN J. BANZHAF AND ROBERT B. GIBSON.

(From the Research Laboratory of the Department of Health, New York City.)

Gravimetric determinations were recorded for the total and several individual proteins of sodium oxalate plasma fractioned

with ammonium sulphate and sodium chloride. When precipitated the plasma-salt solution had a resulting volume of ten times the amount of the plasma employed. Coagulations were on aliquot portions of the filtrates, and the protein constituents (except serum albumin and in part the saturated sodium chloride soluble serum globulin) were calculated by difference. The eleven horses were subjected to an immunization, under Dr. Park's direction, against diphtheria and tetanus toxins simultaneously, each animal subsequently being continued on the toxin to which it responded best. Test bleedings of about 500 cc. only were made until maximum antitoxic potency (with almost coincident greatest variation in content of the proteins) had been attained; afterwards routine bleedings were undertaken. The two refractory, the one medium and the eight horses producing a highly potent antitoxin, showed a maximum increase of from 40 to 114 per cent for the serum globulin (for the refractory horses, 59.3 and 89.8, respectively). In one refractory and one high horse, the serum globulin maximum preceded the highest concentration in antitoxin; with three other horses, both maxima were attained when examined on the second bleeding. In seven of the horses, the greatest increase in serum globulin was coincident with the maximum antitoxic potency. The greatest content in serum globulin was observed in the most potent plasma obtained in the series. The serum globulin subsequently was maintained at a high concentration, roughly paralleling the antitoxic content in the plasma of the individual animals. The serum albumin was diminished a half to a third along with serum globulin increase—though subsequent to the antitoxic and serum globulin maxima, figures as low as a fifth of the original albumin content were noted. The saturated sodium chloride soluble serum globulin was relatively increased from a normal 60 to 80 per cent of the total serum globulin to over 90 per cent; in quantity, there was a rise of over 100 per cent, an increase of 163 per cent being observed in one of the refractory horses. At dilutions of the plasma in the precipitated mixtures of 1 : 1.5, 1 : 5 and 1 : 10, the "euglobulin fraction" amounted to 60 to 70, 20 to 24 and 10 to 15 per cents, respectively, of the total serum globulin in both the normal and antitoxic plasma. In the 850 unit plasma, an increase in the "euglobulin" was noted, but the high content of proteins had

probably influenced the precipitation limits. It would seem then that the "euglobulin" is not increased relatively to the total serum globulin during immunization as has at times been maintained. From the results we have obtained, it may be concluded that in forced immunization, the same characteristic quantitative changes can occur in the blood proteins both of refractory horses and of those yielding a highly potent antitoxin.

THE COMPRESSIBILITY OF GELATIN SOLUTIONS AND OF MUSCLE.

BY LAWRENCE J. HENDERSON AND F. N. BRINK.¹

(From the Laboratory of Biological Chemistry, Harvard Medical School.)

Measurements of the compressibilities of 0.2 per cent and 10 per cent gelatin solutions and of muscle from the foreleg of the rabbit according to the method of Richards² yielded the following results:

Pressure, kilograms per sq. cm.	Compressibilities.		
	0.2 per cent gelatin.	10 per cent gelatin.	Muscle.
100-200	0.000044	0.000041	0.000036
200-300	0.000041	0.000039	0.000034
300-400	0.000039	0.000037	0.000033
400-500	0.000038	0.000036	0.000033
100-500	0.000041	0.000038	0.000034

THE EFFICIENCY OF THE NEUTRALITY REGULATION IN THE ANIMAL ORGANISM.

BY LAWRENCE J. HENDERSON.

(From the Laboratory of Biological Chemistry, Harvard Medical School.)

Theoretically neutrality is most efficiently preserved in solution by acids possessing an ionization constant equal to the square root of the water constant, if only strong bases are present.³ This may be shown mathematically and experiments

¹ Aided by the Elizabeth Thompson Science Fund.

² Richards: Carnegie Institution of Washington, Publication No. 76.

³ This conclusion, developed in a wholly different connection, was presented to the Physico-Chemical Club of Boston and Cambridge, by E. W. Washburn, at the same time that it was presented by me. See E. W. Washburn, *Journal of the American Chemical Society*, xxx, p. 31, 1908.

have verified the theoretical considerations. In the body, however, because of the comparatively small variations in tension of carbonic acid, the bicarbonates are far more efficient than would be such above mentioned substances. A similar consideration probably applies to the phosphates because of the diffusibility of acid phosphates. In great measure the bicarbonates constitute the first reserve and the phosphates the second reserve in neutralizing acid, for the bicarbonates are more efficient at the normal alkalinity of blood, and the phosphates at the exact neutral point.

In the neutralization of acids the compounds of the serum proteins with alkalies play a rôle which, though inferior to that of the above mentioned salts, is not immaterial, nor, at any rate, inconsistent with the idea that to them is mainly due the capacity of blood to give up much of its carbonic acid.

Material increase in the alkalinity of blood, carbonic acid tension remaining normal, would involve such an increase in bicarbonate concentration as materially to increase the osmotic pressure. Such a process would probably be checked by the kidneys.

ON GLYCOSURIA.

By HUGH McGUIGAN.

(From the Laboratory of Physiology and Pharmacology, Washington University, St. Louis.)

In a previous paper (*Amer. Journ. of Physiol.*, xviii, p. 256, 1907) attention was called to the belief that the cause of glycosuria was not due to ferment action but more probably to changes in the activity of the cell protoplasm. If this be true, all work where ferment action is considered has little bearing on the problem. The present work tends to show that changes in the living protoplasm and changes that I believe are intramolecular, are concerned in every case of glycosuria.

Ground muscle or muscle plasma has little influence on glycolysis. Muscular work on the other hand uses up the principal part of the sugar. Gland extracts influence glycolysis but little. The statement of Cohnheim that mixtures of muscle and pancreas extracts cause marked glycolysis, I am unable to confirm. In fact, no such action takes place if bacterial action

is prevented. It can be easily demonstrated however that living muscles utilize sugar.

If the hind legs of an animal be perfused with blood containing sugar there is a marked loss of the sugar. This loss is greater if, at the same time, the muscles are stimulated. The loss is not due to mere filtration as happens when the kidney is perfused, but an actual loss of sugar can be shown. The blood coming from the perfused living legs is dark and venous in color. The sugar destruction continues for some time after the response to electrical stimulation has ceased. The perfusion of a dead leg also shows a loss of sugar, but in this case the loss can be shown to be due to a filtration into the surrounding tissues. The blood retains its arterial color and the leg becomes edematous. Analysis of the tissue shows an accumulation of sugar.

If the liver be perfused there is also a loss of sugar. Only by rapid work and the establishment of the circulation quickly can an accumulation of glycogen be shown. The total carbohydrate in the liver may be more than at the beginning of the perfusion but the increase does not balance the loss from the blood. The sugar destroying power persists after the glycogen storing power has disappeared.

It is clear that the living cell is the active agent in the utilization of sugar. As each cell contains inactive or dead material we may attribute the life processes of the cell to what has been called the biogenic molecule. During life this hypothetical molecule contains chemical groups which have the power of uniting with the sugar. In death there is an intramolecular change and a saturation of the molecules with loss of the power to unite with the sugar.

Loew believes that the activity of the living cell resides in the aldehyde group and the unstable amino group. Pflüger thinks that the cyanogen group is the active agent. Both these theories assume the presence of a doubly bound carbon atom. Both may be true but probably neither is sufficiently comprehensive. Nef's theory of bivalent carbon as applied to organic chemistry includes both and is sufficiently comprehensive to cover the whole field of biological chemical reactions. A polarization of the carbon valencies in all probability will explain the changes to which glycosuria is due.

A COMPARISON OF WAYMOUTH REID'S AND SCHENCK'S
METHODS FOR THE ESTIMATION OF SUGAR IN
BLOOD.

By J. J. R. MACLEOD.

*(From the Laboratory of Physiology and Bio-Chemistry, Western Reserve
University, Cleveland, Ohio.)*

It was shown that comparative estimations of the amount of sugar in blood by the methods of Waymouth Reid and Schenck, gave for the latter a constantly lower value. In most cases the deficit was about 30 per cent. Boiling the blood with Schenck's reagent did not improve the result. Further details regarding these results will be published in the near future.

Eosinophilia and indicanuria. By C. H. NEILSON.

Second Meeting.

Physiology Building. Tuesday morning, December 31. Joint session with the American Physiological Society.

Presiding officers: The President of the American Society of Biological Chemists, Russell H. Chittenden, and the President of the American Physiological Society, William H. Howell.

PROTEIN METABOLISM IN FASTING.

By OTTO FOLIN.

(From the Chemical Laboratory of McLean Hospital, Waverley, Mass.)

A detailed analysis of the urine obtained during a seven day fast from a man whose protein katabolism had previously been reduced to a minimum. The nitrogen elimination rose from day to day during the fast. The conclusion was drawn that the nitrogen elimination during the early stages of fasting can be made high or low at will and in no case do the figures obtained have any bearing on the necessary destruction (or consumption) of protein.

EXPERIMENTAL GLYCOSURIA.

By J. J. R. MACLEOD.

(From the Laboratory of Physiology and Bio-Chemistry, Western Reserve University, Cleveland, Ohio.)

Additional experiments were reported to show that the hyperglycæmia which follows stimulation of the central end of the vagus nerve and stimulation of the spinal cord above the mid-dorsal region does not occur when the partial asphyxia which usually follows such stimulation is prevented by delivering oxygen into the bronchi, and, when necessary, applying artificial respiration with the bellows. Under these conditions no evidence can be obtained of afferent fibers to the so-called diabetic center, nor can efferent fibers be detected in the spinal cord. On the other hand, stimulation of the uncut left great splanchnic nerve is immediately followed by a most marked hyperglycæmia even after all precautions against asphyxia are taken. This hyperglycæmia is accompanied by very marked diuresis and glycosuria, and if the amount of sugar in the blood, the amount in the urine, and the amount of urine excreted per minute be plotted in a curve, a striking parallelism is noted when the stimulation of the splanchnic is kept up for some hours, the amount of sugar in the blood falls off and parallel with it the diuresis and glycosuria. Coincident with this decline in the hyperglycæmia, it is noted that the effect of the stimulation on the arterial blood pressure becomes much less than at first. It is as yet doubtful whether the fibers contained in the greater splanchnic nerve are really glycogenolytic, in the sense that they are secretory, or whether they may be vasoconstrictor to the hepatic artery. In the latter case their stimulation, by constricting this vessel, would cause partial asphyxia of the liver cells by diminishing the arterial blood supply.

FURTHER OBSERVATIONS ON THE PARENTERAL
UTILIZATION OF CARBOHYDRATES

By LAFAYETTE B. MENDEL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University.)

In an earlier communication it was shown that the parenteral introduction of soluble carbohydrates for which the animal organ-

ism possesses no specific digestive enzymes, is followed by the speedy elimination of the injected substance in considerable quantity. Further observations on the behavior of sucrose indicate that the sugar may reappear in the urine in amounts reaching over 90 per cent of that introduced by subcutaneous or intraperitoneal injection in dogs, cats, and rabbits. As yet we have no evidence that the sugar retained is utilized by excretion into the alimentary tract and reabsorption after inversion. An occasional feature of such experiments is the elimination of levorotatory substances, probably conjugated glucuronates. Attempts to induce an adaptation and better utilization of the sugar by repeated parenteral injections have been unsuccessful; neither is there a better utilization in starving animals.

Soluble starch introduced parenterally in rabbits is recovered in part only in the urine, in the form of dextrin-like compounds. The utilization (or retention) is most extensive after subcutaneous injection, less after intraperitoneal, and least after intravenous introduction. Tissue amylases apparently play some rôle in this. A complete utilization, such as Moscotti has claimed for starch suspension, was never observed.

PRELIMINARY REPORT OF CERTAIN INVESTIGATIONS AS TO THE NATURE OF PEPTONES.

By HOWARD D. HASKINS.

*(From the Laboratory of Physiology and Bio-Chemistry, Medical Department
Western Reserve University, Cleveland, Ohio.)*

Hofmeister has recently devised a method for separating and differentiating peptones which promises to greatly advance our knowledge of these substances. Whereas other workers have succeeded in securing but two peptones from any one digestion mixture, his pupils, Stookey¹ and Roper² have separated and studied four or five bodies giving the biuret test (peptones) from a peptic digest of blood protein after all albumoses had been removed.

¹ Stookey: Hofmeister's *Beiträge*, vii, p. 590.

² Roper: *Ibid.*, ix, p. 168.

In the present research a pure protein, recrystallized horse's oxyhemoglobin, was digested by pure pepsin. After completely removing the albumoses, the peptones were fractionally precipitated by Hofmeister's method. A further separation of these fractions was effected by solubility in water, alcohol, ammonium carbonate solution, etc. Six peptone-containing substances were thus secured. The presence of a seventh peptone was detected in the final filtrate after precipitation of the other peptones from the digestion mixture.

As to their reactions, all seven peptones gave the same result as globin to the biuret (positive), Millon (positive), Hopkins-Cole (positive), Molisch (negative), and lead hydroxide (negative) tests. Although containing no loosely-combined sulphur (lead test), two of the peptones yielded considerable sulphur when fused with metallic sodium, and one other showed the presence of a trace of sulphur. Three of the peptones were insoluble in alcohol. Only two gave a precipitate with trichloroacetic acid while all except the seventh peptone were precipitated by tannic acid. The estimation of diamino and monoamino-nitrogen by Hausmann's method gave widely different results in the case of the two peptones that were examined by this method.

THE SPONTANEOUS OXIDATION OF SOME CELL CONSTITUENTS.

By A. P. MATHEWS, O. RIDDLE AND S. WALKER.

(From the Laboratory of Biochemistry and Pharmacology, University of Chicago.)

The fundamental idea underlying this work is that the consumption of oxygen by living matter is the sum of the oxidations of the different substances making up the living mixture. The authors have accordingly been studying the power of various cell constituents to take up oxygen from the air under varying conditions. The method employed (except for the oils which were used pure) was to bring about two grams of the substance dissolved in 50 cc. of alkaline, neutral or acid water into closed flasks of 300 cc. capacity filled with air or oxygen and provided with mercury manometers. The flasks were placed in shaking

machines and the rate of oxidation of the substances measured by the difference in pressure within and without the flasks.

The results thus far obtained are as follows:

(a) *Unsaturated oils.* Linseed oil was used. Confirming Guenthe it was found that linseed oil oxidizes very slowly at first and then at a very much accelerated rate. Autocatalysis occurs. This oxidation is greatly stimulated by violet rays from a mercury lamp, and appears to be retarded by red rays. The last point however is not certain. Sudan III dissolved in the oil suppresses the acceleration due to light. Nicotine greatly retards the oxidation. Morphine chloral hydrate and physostigmine did not affect it. The oxidation is accelerated by the admixture of a little water.

(b) *Sugars.* Sugars burn spontaneously and speedily in alkaline solution. Levulose fastest; galactose a little faster than glucose; glucose, maltose and lactose with the same speed. Fair constants were obtained by monomolecular formula. All sugars except levulose show an accelerated velocity of reaction, proving that the sugars are converted by the alkali into some substance more easily oxidized than the sugar itself. m to $2n$ concentration of alkali oxidizes most rapidly, the rate declining sharply in more dilute or more concentrated solutions. In neutral solutions the oxidation is too slow to detect in several days except with levulose. In acid solutions it is slower. These facts indicate that the sugars act as weak acids. The sugar anion undergoes a rearrangement of its hydroxyls, probably forming a ketone anion which burns rapidly. The oxidizing power of a stronger than $2n$ solution of sodium hydroxide is less than that of m , although the action in the sugar molecule is greater. It is suggested that the glycolytic ferments produce the same rearrangement in the sugar molecule before oxidation that the alkalis produce.

(c) *Protein decomposition products.* Cystein, cystin, tyrosin and leucin were studied. Tyrosin and leucin show no perceptible oxidation in several days in neutral or alkaline solutions.

Cystein in neutral solution rapidly oxidizes itself to cystin. This oxidation is checked by potassium cyanide in a $\frac{1}{100000}$ solution more than 50 per cent and by stronger amounts may be

inhibited for days. The potassium cyanide disappears from the solution. The spontaneous oxidation of cystein is also reduced by chloroform. Further studies in this direction are being carried out.

Cystin in $\frac{n}{2}$ or n sodium hydroxide solution oxidizes rapidly. The substances formed have not been isolated. Small amounts of potassium cyanide inhibit this oxidation for a short time and then enormously accelerate it. The explanation has not yet been found.

The importance of the sulfur group in protoplasm in respiration is thus established. The work is being continued.

THE FATS AND LIPOIDS OF MALIGNANT RENAL HYPERNEPHROMAS.

By H. GIDEON WELLS.

(From the Pathological Laboratory, University of Chicago.)

Four hypernephromas of different type were analyzed as to their content of fatty matter, lecithin and cholesterin. It was found that the typical hypernephromas, with vacuolated cells resembling those of the adrenal and numerous areas of hemorrhage and necrosis, show a striking resemblance to normal adrenal tissue as regards these constituents. Since a hypernephroma of an entirely different type, resembling for the most part a renal papillary carcinoma in its histology, and with cells almost free from vacuolization, showed quite the same proportion of lecithin and cholesterin, it appears that these constituents of the hypernephroma cells are not products of retrogressive processes but are essential constituents of the tumor cells. This affords further evidence of the adrenal origin of these tumors. A specimen showing a structure for the most part like a lipoma, but of a type of hypernephroma already recognized by histo-pathologists, gave chemically an enormous amount of neutral fat; but the amount of lecithin and cholesterin present was far in excess of that found in true lipomas. When recalculated for the dry fat free substance the figures show that this tumor also resembles adrenal tissue in its high content of lecithin and cholesterin. Analysis of two carcinomas and a sarcoma show that this richness in lecithin and cholesterin is characteristic of the hypernephromas.

PROTEIN METABOLISM IN THE DOG.

II. THE INFLUENCE OF LOW CALORIC VALUES OF NITROGEN
ON METABOLISM.

By EMIL OSTERBERG AND C. G. L. WOLF.

(From the Chemical Laboratory, Cornell University Medical College, New York City.)

Animals were carried through a period of eight days of starvation, with subsequent four-day periods of feeding 20 calories, 30 calories, 40 calories, 30 calories and 20 calories of protein (blood albumin), with complete analyses of the urine for the nitrogen and sulphur constituents, for phosphorus and chlorin, and the feces for nitrogen, sulphur and phosphorus. The results show that the retention of sulphur after inanition is relatively very much greater than that of nitrogen. The elimination of ammonia follows pretty well the trend of the nitrogen elimination during periods of protein feeding, but the relationship is much lower than during starvation or when adequate amounts of food containing no nitrogen are administered. During protein feeding the creatinin remains practically constant, with varying amounts of nitrogen ingested. The creatin produced by starvation is inhibited by very small amounts of ingested protein. The undetermined nitrogen follows to a certain extent the amount of protein ingested, but the relationship distinctly falls; so that relatively less undetermined nitrogen is excreted with high protein diet. This is not altogether the case for neutral sulphur. The ethereal sulphur is fairly constant throughout the experiment, and the amount of ethereal sulphur bears no relation to the amount of indican excreted.

Further communication on the effect of vagus inhibition on the output of potassium from the heart.¹ By W. H. HOWELL and W. W. DUKE.

On the chemical study of mental disorders. By WALDEMAR KOCH.

Concerning the pharmacological action of potassium iodide. By L. B. STOOKEY and V. GARDNER.

¹ See the *Amer. Journ. of Physiol.*, xxi, p. 51, 1908.

On the composition of normal lymph from the neck lymphatics of the horse. By J. R. GREER (by invitation).

The presence of glucose in saliva. By J. G. RYAN (by invitation).

The antagonistic action of calcium upon the inhibitory effect of magnesium, with a demonstration.¹ By S. J. MELTZER and JOHN AUER.

Third Meeting.

Kent Chemical Building. Wednesday morning, January 1. Joint session with the Biological Section of the American Chemical Society in affiliation with Section C (Chemistry) of the American Association for the Advancement of Science.

Presiding officers: The President of the American Chemical Society, Marston Taylor Bogert, and the President of the American Society of Biological Chemists and Chairman of the Biological Section of the American Chemical Society, Russell H. Chittenden.

President's address:

SOME OF THE PRESENT-DAY PROBLEMS OF BIOLOGICAL CHEMISTRY.²

By RUSSELL H. CHITTENDEN.

ON THE PASSAGE OF SUBSTANCES INTO THE HUMAN SYSTEM BY OSMOSIS.

By LOUIS KAHLENBERG.

(From the Chemical Laboratory, University of Wisconsin.)

The experiments began with a study of the treatment of cases of blood poisoning by means of saturated aqueous solutions of boric acid. In all cases where such solutions are applied to the thoroughly cleansed skin, boric acid is absorbed and makes its appearance in the urine. The percentage found in the urine rarely reaches more than a few hundredths of 1 per cent.

¹ See the Proceedings (December, 1907, meeting) of the Society for Experimental Biology and Medicine, v, p. 33, 1908.

² See *Science*, xxvii, p. 241, 1908.

A large number of pathological samples of urine were examined, their boric acid content being determined colorimetrically by means of a modification of the turmeric paper method. When the feet of a normal individual are immersed in a saturated boric acid solution up to the ankle, the boric acid appears in the urine in estimable quantity in ten minutes. The presence of traces may be demonstrated after five minutes. Under like conditions not a trace of lithium passes into the system from lithium chloride solutions. Quite weak solutions and also 5 and 10 per cent solutions were tried. Chlorides of calcium and rubidium also do not pass into the system. The tests were made by means of the spectroscope. It is a notable fact that through dead animal or vegetable membranes lithium chloride will pass much more rapidly than will boric acid. And when even a small fraction of a grain of lithium chloride is taken into the mouth, the lithium may be demonstrated to be present in the urine in a very short time. When the feet are soaked in a solution of lithium tetraborate, neither lithium nor boric acid is found in the urine. Thus far all attempts to introduce lithium salts into the system by absorption through the skin have been unsuccessful. On the other hand lithium salts readily make their way through the mucous membranes. Through dead animal and vegetable membranes acids diffuse most rapidly. When the feet are soaked in tenth normal hydrochloric or sulphuric acid the urine, which as is well known is slightly acid in reaction normally, becomes alkaline in five minutes, also scanty as to amount. In ten minutes the alkalinity is much more pronounced. If now the experiment is stopped, the alkalinity persists for three to five hours longer, gradually disappearing. An attempt to secure the same results with citric acid failed, the urine showing if anything a slight increase of acidity. Even a half normal solution of citric acid produced the latter effect. When taken internally the acids mentioned acidify the urine. Thus it appears that when, for instance, sulphuric acid is taken in through the skin quite a different physiological effect is produced from that found when the acid is taken into the digestive tract.

The work demonstrates clearly that living membranes act differently osmotically from dead ones, and that different living membranes may act very differently indeed toward some sub-

stances and yet much the same toward others. Whether a substance will be absorbed or not depends upon its specific nature and that of the membrane. The results of the experiments are a step forward toward a better understanding of the processes of absorption and secretion.

THE ISOLATION AND TOXIC PROPERTIES OF AN ORGANIC SOIL CONSTITUENT.

BY OSWALD SCHREINER AND EDMUND C. SHOREY.

(From the Laboratory of the Bureau of Soils, United States Department of Agriculture, Washington, D. C.)

The authors reported on a crystalline organic compound which had been isolated from several unproductive soils. The compound when tested by physiological methods is harmful to plants and appears to be the cause of the unproductivity in these soils. It can be obtained by extracting the soil with dilute soda solution, acidifying, filtering from the voluminous precipitate of humus bodies produced and shaking the filtrate with ether. The ethereal solution contains the compound and this is obtained in a crystalline form by evaporating the ether on some water. When pure, the compound is white, melts at 98° to 99° and has the composition and other properties of dioxystearic acid prepared by oxidation of elaidic acid.

TOXIC SUBSTANCES ARISING DURING PLANT METABOLISM.

BY OSWALD SCHREINER AND M. X. SULLIVAN.

(From the Laboratory of the Bureau of Soils, United States Department of Agriculture, Washington, D. C.)

For the purpose of studying the factors underlying the decrease in yield of plants grown successively on the same soil, wheat and cowpea were grown on different soils until the yield of the plants became poor. Water extracts of these soils proved to be poor media for the respective plants. On shaking the extract with carbon black, and filtering, they became far better media for plant growth. It must be concluded then

that the soil extracts contained something actually deleterious to the plant since the carbon black made from natural gas acts merely as an absorbing agent.

From wheat-sick soil there was obtained by steam distillation a crystalline body which is toxic to wheat. From cowpea-sick soil in the same manner a crystalline body was obtained which is toxic to cowpea but not to wheat.

BACTERIAL GROWTH AND CHEMICAL CHANGES IN MILK KEPT AT LOW TEMPERATURES.

By M. E. PENNINGTON.

(From the Food Research Laboratory, at Philadelphia, of the Bureau of Chemistry, United States Department of Agriculture.)

Bacteria in milk increased in numbers at a temperature of -0.55° C. Bacterial growth, at the end of a week, was pronounced. There was a steady increase in the number of organisms for five or six weeks, and at their maximum they numbered hundreds of millions. Occasionally they passed the billion mark per cc. This occurred in spite of the fact that, though the milk was never solidly frozen, after ten days to two weeks it was a mass of small ice crystals. Neither odor nor taste indicated the high bacterial content and a curd was not produced, even on heating, until the very end of the experiment.

There were present, at all times during these experiments, acid forming, liquefying and neutral organisms. Acid formers were in lower, and the liquefying organisms in higher, proportion than is commonly found. Certain species, such as *B. formosus*, *B. solitarius* and *B. ravenel*, were especially resistant to cold and frequently were the predominating species or almost in pure culture at the close of the experiment.

Storage at this temperature ordinarily cuts down the number of organisms developing at 37° C., the maximum number being found when the plates were kept at 20° or 0° C.

The acidity increases to such an extent that sometimes 100 cc. of $\frac{1}{16}$ sodium hydrate are required to neutralize 100 cc. of milk, but this acidity has not caused a curd.

A chemical study of the protein of milk in cold storage showed that the casein was rapidly digested until finally more than 30

per cent of it was changed to soluble compounds. Caseoses, amino acid, and probably peptones, increase apparently at the expense of the digested casein. The rapidity with which this digestion takes place varies in different samples but at the expiration of two weeks it is pronounced.

THE CHEMICAL AND THE PHYSIOLOGICAL PROPERTIES OF A SOLUTION OF HYDROCHLORIC ACID AND SODIUM CHLORIDE.

By AMOS W. PETERS.

(From the Zoölogical Laboratory, University of Illinois.)

The influence of neutral salts, e. g., sodium chloride, extends to both chemical properties of solutions and to correlated physiological functions. The killing concentration of hydrochloric acid for protozoa is lowered by the addition of a concentration of sodium chloride which is practically harmless when used alone. This effect can be explained by the increased acidity of the mixed solution, according to Arndt, Euler and others. Colorimetric comparisons with methyl orange, and inversion tests by the polarimetric method, made with the dilute solutions of these experiments, favored this hypothesis, whether the solutions were made with redistilled water or with the original culture liquids. The increased velocity of inversion due to sodium chloride disappeared when the acid products of the reaction demonstrated by Kullgren, increased.

ENDO AND EKTOINVERTASE OF THE DATE.

By A. E. VINSON.

(From the Agricultural Experiment Station, Tucson, Arizona.)

The invertase of the date cannot be extracted by solvents until the fruit ripens. The green date tissues are very active when placed directly in a sugar solution. This is not dependent on the living cell because the inverting action of green tissue is not inhibited by killing mixtures to any greater extent than that of ripe date tissue, where the invertase is soluble. An extensive investigation of the relations of tannin to invertase was made. Tannin

does not retard the inverting action of date tissue or date extracts. It does prevent the solution of invertase in water to a great extent, but the tannin-invertase compound is soluble in glycerin, especially if formed in the presence of glycerin. The invertase of the green date is not soluble in glycerin under any known condition. The portions of the green date which contain no tannin are rich in insoluble invertase. There is no direct relation between the disappearance of soluble tannin and the appearance of soluble invertase other than in point of time.

Since the date is a complex tissue whose inner cells are less protected than yeast cells, and since they cannot glide free among each other when ground and crushed, appreciable numbers should be disrupted by ordinary means for detectable amounts of invertase would escape if it were held by the impermeability of the protoplasmic cell wall alone. The press juices contain those substances which are otherwise retained by the semipermeable *Hautschicht*, but never any invertase. Treatment with ether, chloroform, etc., kills the protoplasm and causes it to shrink away from the cellulose wall, but never liberates the invertase.

After discussing the theory of impermeability of the cell wall to the enzyme in the case of endoenzymes as proposed by Hoppe-Seyler and accepted by Lea, Buchner, Pantanelli and many others the author suggested a modification of Fischer's theory that the enzyme is held by the protoplasm. Insoluble invertase compounds which actively inverted cane sugar were prepared by the author. His theory is as follows: The endoenzyme forms an insoluble combination with some constituent of the protoplasm, of such a character that its catalytic properties are not modified. This compound is independent of the life or death of the protoplasts. It is usually released upon the maturity of the cell, possibly by autolysis, but may be liberated in other cases by external physical or chemical influences. These probably act by destroying the integrity of the cell and allowing contact of substances which are held apart during life.

Application is made of this theory to the observations of several investigators, but notably to those of Buchner on zymase. If zymase is an active, insoluble, protoplasm compound, the results of Buchner may be due to two causes: Some of the compound may have been comminuted so finely as to pass various

filters, and in fact some of Buchner's figures show all but about 10 per cent of the activity of the juice to be removed by filtration, which Buchner attributes to adsorption alone. A second and more probable explanation is that after the integrity of the cell is destroyed by mechanical means (various investigators have shown that the entire protoplasmic content is pressed out) or by protoplasmic poisons, proteolysis of the zymase-protoplasm compound may follow rapidly and free soluble zymase be liberated. It has been shown that further proteolysis destroys the zymase and that the zymase may be protected by added protein such as blood serum. The first step in the proteolysis of the zymase-protoplasm compound would be the removal of the protoplasmic portion.

GLYCOCOLL AS A PRODUCT OF URICOLYSIS.

By L. B. STOOKEY.

(From the Laboratory of Physiology, University of Southern California.)

It is well known that *in vitro* uric acid may be broken down by appropriate means into glycocoll. Whether or not glycocoll is formed in the animal body as an intermediary product of uricolysis is unsettled.

To throw light on this problem mixtures of hashed liver and hashed kidney to which were added physiological saline containing 0.2 per cent benzoic acid and 0.2 per cent uric acid were incubated varying lengths of time and the content of hippuric acid determined. In every case the greater portion of uric acid was destroyed.

Control experiments without the addition of uric acid showed approximately the same formation of hippuric acid.

From these results it might seem that under the conditions employed, uric acid is not broken to any appreciable extent into glycocoll.

A STUDY OF THE INFLUENCE OF POTASSIUM CYANIDE
ON THE EXCRETION OF NITROGENOUS SUBSTANCES
IN THE URINE OF DOGS.

By WILLIAM H. WELKER.

*(From the Laboratory of Biological Chemistry of Columbia University, at the
College of Physicians and Surgeons, New York.)*

Long continued and carefully controlled experiments on six dogs, on moderate or comparatively low or high planes of protein nutrition, failed to show definite alterations of the partition of nitrogen among the urinary constituents, after subcutaneous dosage with potassium cyanide. The doses of the cyanide varied between one-half and $3\frac{1}{2}$ milligrams per kilo, per day, for periods ranging between 4 and 12 days. Total nitrogen, ammonia, urea, allantoin, uric acid, purin bases and creatinin were quantitatively determined continuously. The results may mean that the doses used did not regularly modify oxidation or any other important metabolic processes in the particular experiments already completed.

A demonstration of a method (with apparatus) of showing the electric charges of colloids. By A. B. MACALLUM.

The protein of tumors. By S. P. BEEBE.

Iodic determination in the thyroid gland. By S. P. BEEBE and L. W. RIGGS.

On the action of nitric acid on nucleic acids. By WALTER JONES.

On the occurrence of a phytin splitting enzyme in animal tissues. By E. V. MCCOLLUM and E. B. HART.

Nitrogen in protein bodies. By THOMAS B. OSBORNE and C. A. BRANTLECHT.

Fourth Meeting.

Physiology Building. Thursday morning, January 2.

Presiding officers: The President, Russell H. Chittenden, and the Vice-President and President-elect, John J. Abel.

THE EXTRAINTESTINAL ORIGIN OF HYDROBILIRUBIN.

By A. E. AUSTIN AND MABEL D. ORDWAY.

(From the Laboratory of Medical Chemistry, Tufts College, Boston, Mass.)

The enterogenous origin of stercobilin and urobilin, which we regard as identical, from bilirubin by reduction and hydration in the intestine through the action of bacteria is fully established by the following facts: the urine of the new born contains no urobilin on account of the absence of bacteria in their intestinal tract (S. Gerhardt) and is scantily present in fasting where the secretion of bile is limited. When the ductus choledochus is tied urobilin disappears from the urine and bilirubin takes its place; while little or no stercobilin is found in the feces. Now if bilirubin or bile be ingested urobilin returns to the urine and the feces are again stained with this pigment (Quinke and F. V. Miller). When a biliary fistula is established urobilin is found in the bile; when the common duct is tied this pigment disappears from the fistula bile (A. Beck). To this is opposed the results of Fischer who succeeded, after tying the duct and freeing the urine wholly from urobilin and the feces almost entirely, by the injection of amyl alcohol and other blood destroying agents, in causing the flow of a bile containing urobilin.

This undoubtedly is the physiological process of formation of urobilin but there are various circumstances which preclude this as the sole method of its formation, among which are the following: urobilin has been found in hemorrhagic ascitic fluid in the abdomen where as demonstrated at the autopsy and by the absence of this pigment in the feces, no bile could enter the intestine on account of cancer of the gall bladder involving the duct (S. Gerhardt); hydrobilirubin has been found in thrombi and hemorrhagic effusions into intercellular tissue (Hayem); this pigment has been found in blood serum containing blood pigment where bacteria were entirely absent (Winter); bilirubin can be converted to hydrobilirubin in autolysis of the liver (Magnus-Levy).

In order if possible to add to our knowledge of this subject we examined fifty-seven specimens of fistula bile from as many different individuals, on the first, second and third day after opera-

tion; the feces, urine or both were examined at the same time. Of these only seven showed absence of this pigment in feces or urine; of the other fifty, forty-six had urobilin in the bile. Of the seven without this pigment in urine or feces only two had urobilin in the bile. In order to explain this apparent extraintestinal origin, since we may regard the absence of this pigment in feces or urine as proof that no bile entered the intestine, several theories have been offered of which at present we shall consider only two: the healthy liver has the power of converting urobilin into bilirubin by oxidation; the diseased liver has lost this power (Vitali). All our efforts at oxidizing urobilin by mild oxidizing agents like peroxide of hydrogen failed, hence the process is more than a mere oxidation, if it exists.

The tissues and blood can convert bilirubin into urobilin; by placing varying quantities of bilirubin in sterile blood drawn from the umbilical cord and protected by toluol, we attempted to effect this transformation, but failed. It is still left us to attempt this by perfusion of tissues with blood containing bilirubin.

ON THE ALLEGED FORMATION OF BILE PIGMENTS AND BILE ACIDS BY THE ACTION OF TRYPSIN ON HÆMOGLOBIN.

By FREDERICK S. HOLLIS (by invitation).

(*From the Laboratory of Medical Chemistry, Tufts College, Boston, Mass.*)

In his two articles on the subject A. C. Croftan¹ states that the formation of bile acids and bile pigments is a purely chemical process due to the action of trypsin on hæmoglobin in the presence of dextrose, and not resulting from any specific activities of the liver cells. He obtained by the action of an extract of liver cells, which were shown to contain trypsin and had been kept under absolute alcohol for two weeks, and also by the action of trypsin on hæmoglobin, in the presence of dextrose, substances which gave tests for bile acids and bile pigments. Thy-

¹ Croftan: *Philadelphia Med. Journ.*, pp. 78 and 142, 1902, also *Archiv. für die ges. Physiol.*, xc, p. 685, 1902.

it was excreted in quantity both in the bile and pancreatic juice. Hehner's test for formaldehyde was used; and by rough colorimetric comparison it was estimated to be equivalent to a 1:12000 solution of formaldehyde. It was shown to be present in bile contained in the gall-bladder twenty-four hours after fifteen grams of hexamethylentetramin had been given by mouth.

Bacteriological observations were made on patients with biliary fistulæ before and after giving the drug; and in every case the bile became sterile within one to five days when the dose of hexamethylentetramin was 75 grains a day or more. As in the urinary bladder they appear again as the dose is decreased.

The bile discharging through the fistulæ, when acidified and distilled, always gave the test for formaldehyde, the amount present varying with the dose.

In every case the patient's general condition improved. The discharging bile changed from a dirty, turbid fluid to the golden yellow color of the normal bile; and the fistulæ healed rapidly.

It was repeatedly shown to be present in the cerebro-spinal fluid, even after small doses by mouth. In one case with a badly infected cerebro-spinal fistula, with sloughing and a purulent discharge, the organisms gradually disappeared after hexamethylentetramin was administered; the fistula closed, and the patient made a good recovery.

It was also present in the pus from a gonorrheal arthritis but sufficient time has not as yet elapsed for a report on its therapeutic effect in this case.

THE DIGESTIVE GLAND OF THE CRAWFISH.

By H. C. BRADLEY (by invitation).

(From the Laboratory of Physiological Chemistry, University of Wisconsin.)

In extracts of the digestive glands of the common crawfish, *Cambarus*, are found enzymes which correspond to the following in vertebrates: trypsin, erepsin, amylopsin, steapsin, invertase, maltase, tyrosinase. Milk curdles rapidly in the presence of the juice. Pepsin, lactase, citase, and chitinase are apparently wanting.

The gland is usually pigmented a vivid green. This pigment can be extracted with boiling acid alcohol, with glacial acetic

acid, and by dilute alkali solutions. It gives no absorption bands in the spectroscope, does not react with Ehrlich's solution, and gives the following positive reactions for biliverdin: Gmelin's test, Huppert's and Hammarsten's reactions, and a reaction proposed by Jollé depending upon the use of Hübl's iodine solution. To eliminate the fats, lipochromes, etc., the brei, or watery solution of the pigment, is precipitated with calcium chloride and sodium hydroxide, filtered, and the precipitate extracted with boiling alcohol and ether. The lipochromes, lutein and crustaceorubin, are thus completely removed along with lipoids and soaps. If the precipitate is digested with acid-alcohol a typical Huppert's reaction results, and the alcoholic solution of the pigment seems to be identical with biliverdin. It may be reprecipitated as a calcium salt and set free again by acid alcohol.

The presence of a bile pigment in the "liver" of this animal is of interest since neither blood nor muscle tissue contains the usual precursor, hæmoglobin.

ON THE EFFECT OF CERTAIN CONDITIONS UPON POSTMORTEM AUTOLYSIS.

BY HOLMES C. JACKSON.

(From the Laboratory of Physiological Chemistry, Albany Medical College.)

These experiments were conducted with the view to determining the conditions which occasioned the "latent period" in liver autolysis noticed by Claypon and Schryver, and by varying these conditions, to obtain if possible a further deference of the commencement of autolysis. The conditions were varied by the removal of the blood by washing the organ *in situ* with normal salt solution; by changing the reaction of the tissue by means of acid and alkaline phosphates and alkaline Locke's solution; by shutting out the presence of light; by variations in temperature; and by varying the condition of the cells of the tissue by overfeeding and by starvation. The experiments were all carried out under absolutely aseptic precautions, no antiseptics being employed except in the study of their effect as compared with the controls. In each experiment the autolytic mixture

under consideration was shown to be sterile by agar slants and stained smears. The acidity to phenolphthalin and alkalinity to dimethylaminoazobenzol, the depression of the freezing point and the non-coagulable nitrogen were determined in the boiled filtrate from samples removed from the general mixture at definite periods, usually every hour or hour and a half during the autolysis. The study embraces the changes occurring in the first six to twelve hours after the removal of the liver from the body of the animal.

The following statements comprise the results obtained from 30 experiments.

(1) Toluol in amounts ordinarily employed for antiseptic autolysis diminishes the autolysis as shown by the increase in the depression of the freezing point Δ , by about 25 per cent and as indicated by the increase in non-coagulable nitrogen, by about 50 per cent.

(2) Hepatic autolysis was greatest in the animals which had been well-fed for some days previous to the experiment. In the case of starving animals the autolysis only amounted to about 75 per cent of that observed in the organs of the well-fed animals. This is the reverse of that observed by Claypon and Schryver.

(3) With well-fed animals, no difference in the rate of autolysis could be determined whether the organ contained blood or not (washed *in situ* with salt solution). In the starved animals, however, the autolysis as indicated by the non-coagulable nitrogen and increased acidity to phenolphthalin was greater by 300 per cent in the washed than unwashed organs (cf. Pearce and Jackson).

(4) Experiments in which the organs were removed from the animal and in which autolysis was allowed to proceed in the dark showed no difference in results from the controls carried on in the light.

(5) The presence of disodium hydrogen phosphate or dihydrogen sodium phosphate in 5 per cent solutions apparently does not alter the rapidity of the autolysis to any great degree. The tissue, in the course of one to two hours, attempts to reestablish its neutral condition (Henderson) as indicated by the relation of the figures obtained in titrating with the dimethylaminoazobenzol and with phenolphthalin.

(6) The fact of the latent period of two to four hours observed by Claypon and Schryver in well-fed animals, could not be substantiated as these authors found it. A period during which autolysis was not evident did appear at times but it was usually in the animals which had been starved some days previous to the experiment. The temperature of the diluting fluid (usually salt solution) plays some part in the appearance of the "latent" period. If the salt solution is warmed when the ground tissue is added to it there is less of a tendency for the autolysis to be delayed.

(7) In the case of Locke's solution to which sodium carbonate had been added to increase its alkalinity to 0.5 per cent, no alteration in autolytic activity could be observed.

THE PEROXIDASE REACTION OF MILK.

BY J. H. KASTLE AND M. B. PORCH.

(From the Hygienic Laboratory, United States Public Health and Marine Hospital Service, Washington, D. C.)

As the result of an examination of a large number of samples of milk by means of three peroxidase reagents it has been found that the peroxidase reaction of raw milk (cow's) is extremely variable, and that as ordinarily carried out this reaction is an unsafe criterion whereby to determine whether a given specimen of milk has been sterilized by heat.

In this connection, a number of substances, especially among the aromatic phenols, have been found to greatly intensify the peroxidase reaction of milk. Among these may be mentioned phenol itself, the three cresols and beta-naphthol. Through the use of such substances it has been found possible by means of the ordinary peroxidase reagents, viz: guaiacum, phenolphthalin, and paraphenylene diamine, to readily and certainly distinguish between raw milk and that which has been sterilized by heat, at temperatures higher than 60° C.

The system (peroxidase, hydrogen peroxide and phenol) has been found to exhibit many analogies to the oxidases. This in itself is an interesting phase of the subject under consideration and will be further investigated.

It has also been found that while milks which have been heated to 70° C. for one hour or 75° C. for twenty minutes no longer give the peroxidase reaction, this reaction is not diminished but, if anything, somewhat intensified by heating the milk to 60° C. for twenty minutes. It is evident, therefore, that sterilization at 60° C. for twenty minutes as recently recommended by Rose-nau and McCoy, on the basis of their bacteriological studies, does not destroy the biological properties of milk, at least in so far as the presence of the peroxidases affords evidence of biological activity.

Finally, it has been observed that the milks of different cows of the same herd exhibit considerable differences in peroxidase activity, and that towards the three peroxidase reagents which have been investigated, woman's milk shows decidedly less peroxidase activity than cow's milk. For cow's milk the peroxidase reaction, especially under the sensitizing influence of certain of the phenols, is constant; for woman's milk, on the other hand, this reaction is in many cases still uncertain, depending for its exhibition on properties of the milk which at present are but imperfectly understood. As a rule, however, it has been found that human milks during the colostrous stage of lactation possess greater peroxidase activity than milks exhibiting no colostrous properties. This is in harmony with the experience of other investigators, among them Thiemich, Marfan and Gillet.

THE EFFECT OF CASTRATION ON METABOLISM.

By FRANCIS H. MCCRUDDEN

(From the Laboratory of Biological Chemistry, Harvard Medical School.)

This research was undertaken to determine, by complete analysis of the food, feces and urine, the effect of castration on the metabolism of the adult animal. Four dogs, two males and two females, were used for the experiment.

A metabolism experiment of twenty days was carried out on each of the animals. They were then castrated, and after the wounds healed, they were each subjected again to a metabolism experiment of twenty days. The food consisted of ox heart, lard, cracker dust and water in the proportion of twenty grams

meat, five grams cracker and three grams lard per kilo body weight per day. The urine, food and feces were analyzed quantitatively for nitrogen, sulphur, calcium, phosphorus and magnesium.

Results. The most marked effect of the operation was a very greatly increased catabolism of nitrogen, sulphur, phosphorus and magnesium. There was no effect on the metabolism of calcium. The results will be published in detail later.

EXPERIMENTS UPON THE METABOLISM OF PHOSPHORUS IN MAN.

By H. C. SHERMAN.

(From the Havenmeyer Chemical Laboratory, Columbia University.)

The data of intake and output of phosphorus in sixteen metabolism experiments¹ upon man by the author and 65 experiments from the literature have been arranged for comparison in order of the amounts of phosphorus contained in the food. Most of the experiments are of short duration and the gain or loss shown is undoubtedly influenced by the habitual level of phosphorus metabolism. The data, therefore, do not show the minimum requirement, but do indicate the average amount required for the maintenance of phosphorus equilibrium at the normal level of a full diet to be about 1.5 grams of phosphorus or 3.5 grams of P_2O_5 per day. With larger amounts in the food the output tends to increase with the intake, as in the case of the nitrogen metabolism. When, however, the food furnishes over 2.5 grams of phosphorus (about 5.75 grams of P_2O_5) per day there is usually a marked storage of phosphorus in the body.

In man the distribution between feces and urine of the excreted phosphorus is so variable that no inferences regarding phosphorus balance should be drawn from any experiments except those in which the output by both feces and urine is carefully determined.

The work forms part of an investigation undertaken in cooperation with the United States Department of Agriculture, and will

¹ The data of some of these experiments have been given in bulletins of the United States Department of Agriculture but without full discussion.

be published in full by the Office of Experiment Stations of that Department.

ON TURGOR PRESSURE IN WOUNDED PLANT TISSUES.

By HERBERT M. RICHARDS.

(From the Botanical Laboratory, Barnard College, Columbia University.)

Some years ago the writer carried on a number of experiments as to the turgor relations of wounded tissue, in comparison with the normal state. The work has never been fully completed but some definite results, that are not without interest, were obtained. It is known that wounding in plant tissue is followed by increased respiration and by a rise in temperature of the wounded parts which extends some little distance from the wounded surface itself. The curves of respiration and of temperature increase are approximately the same and attain their maximum about twenty-four hours after wounding, under normal atmosphere and at a temperature of about 20° C.

The question at hand is as to whether these changes are accompanied by alteration of turgor pressure of the cells in the immediate neighborhood of the injury. Various plants were experimented with, the traumatic respiration curve of which is known in some cases but not in others. Onions, carrots, beets, radishes, the leaves of begonia, of ficus and of elodea were all used. Most of them gave results, but the most favorable was the onion. This bulb with its relatively large amount of stored food material, which is present in a very readily available form, has a very high traumatic respiration. Various forms of wounds were made and the isotonic values of both the injured specimens and of the uninjured controls were determined. The normal turgor pressure in terms of potassium nitrate solution was from 3.5 to 4 per cent of that salt in the specimens experimented with, although it may be said that cane sugar was the plasmolytic agent most commonly employed. During what must have been the height of the respiration curve, the turgor pressure fell to about 3 to 3.5 per cent potassium nitrate solution, a difference, that is, of 0.5 per cent. This fall was pretty regular and always about the same. The natural healing processes which follow were accompanied by

a reassumption, to a greater or less extent, of the original turgor pressure, though the recovery of the turgor is not as rapid as is the recovery of the normal respiration and four or five days elapsed before it was complete. During the experiments the plants were protected from change in atmospheric conditions as far as possible, being kept in a moist atmosphere and at as constant a temperature as possible. The carrots and beets responded similarly and also the begonia and ficus leaves though, as might be expected, to a lesser extent. The elodea alone gave no result at all.

As an explanation of the observed phenomena it is suggested that the fall in turgor was due to the exhaustion from the cells, during the period of increased respiration, of some of the osmotically active substances—possibly sugars—owing to the increased demands of the accelerated catabolic processes.

THE PROBABILITY OF A RADIOTROPIC RESPONSE.

By C. STUART GAGER.

(From the New York Botanical Garden.)

Tropistic movements of organisms are due to a unilateral distribution of some environmental stimulus, such as light, water, gravity, etc. The failure of the author and of other workers to secure a tropistic movement toward or from the rays of radium is explained here on the hypothesis that the rays are too penetrating to be felt, as a stimulus, unilaterally. If the conditions of experimentation can be so arranged that the rays whose penetrating power is nearly spent can reach the organism, then they may fail to pass clear through the exposed organ, and thus a tropistic movement may be called forth. Thus far however no successful results have been reported. By interposing a screen of water between growing roots and glass tubes containing radium bromide, the author has been able to observe a marked curvature toward the radium, but the result needs further confirmation before it can be positively attributed to the influence of the radium rays.

A FURTHER STUDY OF SOLUTION TENSION AND TOXICITY IN LIPOLYSIS.

By RAYMOND H. POND.

(From the New York Botanical Garden.)

A prior study of the lipolysis of ethylbutyrate showed very clearly that the toxicity of a series of metallic nitrates does not vary directly as the solution tension of those salts. In the lipolysis of ethylacetate, I am also unable to find any direct relation between solution tension and toxicity. The results moreover justify the conclusion that the zymolyte itself is a factor in relative toxicity. The relative toxicity of a series of salts is not the same when the zymolyte is ethylbutyrate as when the zymolyte is ethylacetate. The concentration of the enzyme was also found to be a factor.

NOTES ON THE CHEMICAL NATURE OF EGG CASES OF TWO SPECIES OF SHARKS.

By LOUIS HUSSAKOF AND WM. H. WELKER.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Egg cases of the skate (*Raja crinacea*) and the Port Jackson shark (*Heterodontus japonicus*) were examined. Peptic digestion, even when continued for 24 to 36 hours, did not visibly affect the substance of the skate capsules. Subsequent pancreatic digestion was also without material effect. Washed with water, alcohol and ether, and dried over sulfuric acid, the residues from the skate cases could be readily reduced to a fine grayish black powder, which failed to go into solution in such liquids as chloroform, but which dissolved in cold dilute and strong aqueous solutions of acids or alkalis. Such solutions were deep red in color. The material contained nitrogen and sulfur, but was free from phosphorus. Neither sulfate nor reducing substance was obtained by hydrolysis. The powder responded readily to various protein color tests, but failed to yield the α -naphthol test for carbohydrate. The main bulk of the substance comprising the cases resembles keratin, but is more soluble than the latter.

The coloring matter in alkaline solutions of the skate cases was freed from extraneous matter by neutralization. The colored filtrate was thoroughly dialyzed. The pigment was indiffusible. The dialyzed liquid yielded, on desiccation, glistening, translucent, reddish black scales. The latter were soluble in water, as well as in acid and in alkaline aqueous solutions. Solutions of the pigment are delicately responsive to change of reaction—acid produces a straw yellow; alkali, a dark brown.

Similar results were obtained with the shark cases. Additional observations will shortly be reported.

A COMPARATIVE STUDY OF THE HYDROLYSIS OF DIFFERENT PROTEINS IN PEPSIN-ACID SOLUTIONS.

By WILLIAM N. BERG.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

The method of the investigation was described in a previous publication.¹ Thus far the following proteins have been used: fibrin, elastin, edestin, acid albumin, alkali albuminate, egg albumin and yeast nucleoprotein. The following acids, in concentrations normal to a 0.2 per cent solution of hydrochloric acid ($\frac{N}{18.229}$) were used; hydrochloric, nitric, sulfuric, phosphoric, oxalic, tartaric, citric, lactic, acetic and boric.

The results obtained indicate that the above named proteins do not digest with equal speeds under the conditions of the experiments. In hydrochloric acid, edestin digests most rapidly, and then follow, in the order given, alkali albuminate, acid albumin, fibrin, egg albumin, nucleoprotein and elastin. This order, with few exceptions, is practically the same for most of the acids. In hydrochloric acid all of these proteins digested more rapidly than in any other acid; the order of the acids in efficiency being usually as follows: hydrochloric, nitric, oxalic, phosphoric, sulfuric, tartaric, lactic, citric, acetic and boric. The chief variant from this sequence was sulfuric acid.

¹ Berg and Gies: *Jour. Biol. Chem.*, ii, p. 489, 1907.

FURTHER OBSERVATIONS ON PROTEIN SALTS.

By WILLIAM J. GIES.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.)

Various salts of mucoids, nucleoproteins and alkali albuminates have been made by dissolving the pure protein in dilute alkali and dialyzing away the excess of the latter. After removal of the excess of alkali in this way, until the solution no longer exhibits alkalinity to phenolphthalin, but is acid to the latter, the solution is perfectly clear and usually manifests distinct alkalinity to litmus. But continued dialysis of the nucleoprotein and alkali albuminate solutions for three or four days more causes the solution gradually to become amphoteric to litmus and finally wholly acid to this indicator; and an increasing proportion of the protein is deposited meanwhile. All the salts thus far obtained appear to be somewhat diffusible and exert comparatively high osmotic pressures. Ash content in the material obtained from dialyzed solutions by drying at 40° C. varied usually from 7 to 10 per cent.

Messrs. Welker and Crawford obtained the following preliminary data for conductivity of some mucoid salts after dialysis for four months in the constant presence of toluol.

Mucoid salt.	Amount added to 100 cc. H ₂ O gram.	Amount Actually dissolved in 100 cc. H ₂ O gram.	Conductivity in reciprocal ohms $\times 10^6$	
			Observed.	Approximately calculated, per gram in 100 cc. H ₂ O.
Potassium.....	0.2	0.2(?)	186.0	930
Sodium.....	0.2	0.176	141.8	807
Calcium.....	0.2	0.112	73.1	653
Barium.....	0.2	0.083	57.9	698

The work is going forward with the coöperation of Messrs. Crawford, Steel, Eddy, Herzfeld, Davis and Lothrop. At present various methods of preparation and their effects are being carefully studied.

Further studies on the application of Folin's creatin and creatinin methods to meats and meat extracts. By A. D. EMMETT and H. S. GRINDLEY.

The determination of ammonia in meat and meat products. By

F. W. GILL and H. S. GRINDLEY.

The blood clot of *Limulus*. By CARL L. ALSBERG.

On the decomposition of nucleic acids by nuclease. By WALTER JONES.

See page xlviii for the joint recommendations of the Physiological and Biochemical Committees on Protein Nomenclature, which were ordered to be printed by the American Physiological and American Biochemical Societies in joint session, December 31, 1907.

JOINT RECOMMENDATIONS OF THE PHYSIOLOGICAL AND BIOCHEMICAL COMMITTEES ON PROTEIN NOMENCLATURE.

Since a chemical basis for the nomenclature of the proteins is at present not possible, it seemed important to recommend few changes in the names and definitions of generally accepted groups, even though, in many cases, these are not wholly satisfactory. The recommendations are as follows:

First: The word *proteid* should be abandoned.

Second: The word *protein* should designate that group of substances which consists, so far as at present is known, essentially of combinations of α -amino acids and their derivatives, e. g., α -amino acetic acid or glycocoll; α -amino propionic acid or alanin; phenyl- α -amino propionic acid or phenylalanin; guanidin-amino valerianic acid or arginin, etc., and are therefore essentially polypeptids.

Third: That the following terms be used to designate the various groups of proteins:

I. SIMPLE PROTEINS. Protein substances which yield only α -amino acids or their derivatives on hydrolysis.

Although no means are at present available whereby the chemical individuality of any protein can be established, a number of simple proteins have been isolated from animal and vegetable tissues which have been so well characterized by constancy of ultimate composition and uniformity of physical properties that they may be treated as chemical individuals until further knowledge makes it possible to characterize them more definitely.

The various groups of simple proteins may be designated as follows:

a. *Albumins*. Simple proteins soluble in pure water and coagulable by heat.

b. *Globulins*. Simple proteins insoluble in pure water but

soluble in neutral solutions of salts of strong bases with strong acids.¹

c. Glutelins. Simple proteins insoluble in all neutral solvents but readily soluble in *very* dilute acids and alkalies.²

d. Alcohol-soluble proteins. Simple proteins soluble in relatively strong alcohol (70 to 80 per cent), but insoluble in water, absolute alcohol, and other neutral solvents.³

e. Albuminoids. Simple proteins which possess essentially the same chemical structure as the other proteins, but are characterized by great insolubility in all neutral solvents.⁴

f. Histones. Soluble in water and insoluble in very dilute ammonia and, in the absence of ammonium salts, insoluble even in an excess of ammonia; yield precipitates with solutions of other proteins and a coagulum on heating, which is easily soluble in very dilute acids. On hydrolysis they yield a large number of amino acids among which the basic ones predominate.

g. Protamins. Simpler polypeptids than the proteins included in the preceding groups. They are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties and form stable salts with strong mineral acids. They yield comparatively few amino acids, among which the basic amino acids greatly predominate.

II. CONJUGATED PROTEINS. Substances which contain the protein molecule united to some other molecule or molecules otherwise than as a salt.

a. Nucleoproteins. Compounds of one or more protein molecules with nucleic acid.

¹ The precipitation limits with ammonium sulphate should not be made a basis for distinguishing the albumins from the globulins.

² Such substances occur in abundance in the seeds of cereals and doubtless represent a well defined natural group of simple proteins.

³ The sub-classes defined (*a, b, c, d*) are exemplified by proteins obtained from both plants and animals. The use of appropriate prefixes will suffice to indicate the origin of the compounds, e. g., ovoglobulin, myoalbumin, etc.

⁴ These form the principal organic constituents of the skeletal structure of animals and also their external covering and its appendages. This definition does not provide for gelatin which is however an artificial derivative of collagen.

b. Glycoproteins. Compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid.

c. Phosphoproteins. Compounds of the protein molecule with some, as yet undefined, phosphorus containing substance other than a nucleic acid or lecithin.¹

d. Hemoglobins. Compounds of the protein molecule with hematin or some similar substance.

e. Lecithoproteins. Compounds of the protein molecule with lecithins (lecithans, phosphatids).

III. DERIVED PROTEINS.

1. *Primary Protein Derivatives.* Derivatives of the protein molecule apparently formed through hydrolytic changes which involve only slight alterations of the protein molecule.

a. Proteans. Insoluble products which apparently result from the incipient action of water, very dilute acids or enzymes.

b. Metaproteins. Products of the further action of acids and alkalies whereby the molecule is so far altered as to form products soluble in very weak acids and alkalies but insoluble in neutral fluids.

This group will thus include the familiar "acid proteins" and "alkali proteins," not the salts of proteins with acids.

c. Coagulated proteins. Insoluble products which result from (1) the action of heat on their solutions, or (2) the action of alcohols on the protein.

2. *Secondary Protein Derivatives.*² Products of the further hydrolytic cleavage of the protein molecule.

a. Proteoses. Soluble in water, uncoagulated by heat, and precipitated by saturating their solutions with ammonium sulphate or zinc sulphate.³

b. Peptones. Soluble in water, uncoagulated by heat, but not

¹ The accumulated chemical evidence distinctly points to the propriety of classifying the phosphoproteins as conjugated compounds, i. e., they are possibly esters of some phosphoric acid or acids and protein.

² The term secondary hydrolytic derivatives is used because the formation of the primary derivatives usually precedes the formation of these secondary derivatives.

³ As thus defined, this term does not strictly cover all the protein derivatives commonly called proteoses, e. g., heteroproteose and dysproteose.

precipitated by saturating their solutions with ammonium sulphate.¹

c. Peptids. Definitely characterized combinations of two or more amino acids, the carboxyl group of one being united with the amino group of the other, with the elimination of a molecule of water.²

RUSSELL H. CHITTENDEN,	} <i>For the American Society of Biological Chemists.</i>
OTTO FOLIN,	
WILLIAM J. GIES,	
WALDEMAR KOCH,	
T. B. OSBORNE,	
T. B. OSBORNE,	} <i>For the American Physiological Society.</i>
P. A. LEVENE,	
J. A. MANDEL,	
A. P. MATHEWS,	
LAFAYETTE B. MENDEL,	

Chicago, December 31, 1907.

¹In this group the kyrins may be included. For the present we believe that it will be helpful to retain this term as defined, reserving the expression peptid for the simpler compounds of *definite* structure, such as dipeptids, etc.

²The peptones are undoubtedly peptids or mixtures of peptids, the latter term being at present used to designate those of definite structure.

VIII. RESEARCHES ON PYRIMIDINS: A METHOD OF SEPARATING THYMIN FROM URACIL.

(Twenty-ninth Paper.)

By TREAT B. JOHNSON.

(From the Sheffield Laboratory of Yale University.)

(Received for publication, March 28, 1908).

New data about the cleavage products of nucleic acids contribute to our knowledge of the constitution of these acids. Practical analytical methods for the quantitative determination of the products of hydrolysis are of the greatest importance. Kossel and his co-workers have developed excellent methods for determining the purin bases—xanthin, guanin, adenin and hypoxanthin. On the other hand, methods for separating quantitatively the pyrimidins are lacking. A delicate qualitative test for uracil and cytosin, in presence of thymin, has been described in a publication from this laboratory,¹ but at present there is no test, or characteristic derivative known, which serves for the identification of thymin in presence of uracil. For its detection we make use of the sublimation, the behavior towards silver nitrate and its elementary analysis.

A careful review of the nucleic acid literature reveals the fact that several investigations, in this field, signify the necessity of a practical method of separating thymin from uracil. Kutscher² in an investigation on the autolysis of thymus glands isolated 0.6 gram of a crystalline substance whose properties corresponded with those of thymin, but whose content of nitrogen did not agree with the theoretical value. He found 23.4 and 24.1 per cent of nitrogen while the calculated value for thymin is 22.22 per cent. His views are summarized in his own words: "Diese Reactionen der Krystalle sowie ihr Verhalten gegen ammoniakalische Silberlösung sprachen für Thymin, doch

¹Wheeler and Johnson: *This Journal*, iii, p. 183.

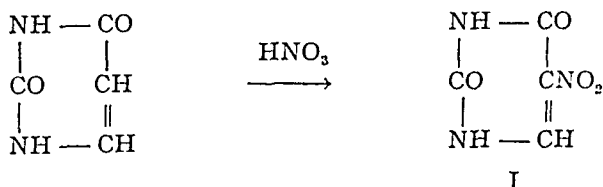
²*Zeitschr. f. physiol. Chem.*, xxxiv, p. 114.

bestätigte die Analyse diese Voraussetzung nicht völlig. Auch durch vielfache Umkrystallization und andere Reinigungsversuche liess sich dieser Stickstoffwerth nicht herunterdrücken. Es musste also dem Thymin eine stickstoff reichere Substanz vielleicht Uracil beigemischt sein."

Steudel¹ in an investigation on the hydrolysis of thymus nucleic acid with hydriodic acid isolated 5.9330 grams of a substance which gave 23.38 per cent of nitrogen on analysis. He says: "Als die Flüssigkeit jetzt eingeengt wurde, schied sich ein Gemenge von Thymin und Uracil aus, das sich in Ermangelung einer guten Trennungsmethode nicht weiter aufgeteilt, sondern als solches analysiert habe." He² also obtained a mixture of thymin and uracil in a later investigation on the oxidation of nucleic acid with nitric acid. In a recent paper, entitled "Die Zusammensetzung der Nucleinsäuren aus Thymus und aus Heringssperma," Steudel³ wrote: "Der nach der Krystallization der alloxurbasen noch bleibende Rest lieferte weiterer Behandlung Thymin und Uracil. Diese beiden Körper liessen sich durch fraktionierte Krystallization zwar nicht quantitativ aber doch qualitativ gut trennen."

I shall describe in this paper a new, characteristic derivative of thymin, and a method of separating, practically quantitatively thymin from uracil.

Uracil and thymin do not react, below 100°, with nitric acid of density 1.41. On the other hand uracil dissolves, at ordinary temperature, in fuming nitric acid of density 1.5 giving practically a quantitative yield of 5-nitouracil I. Thymin reacts, under the same conditions, giving a quantitative yield of the addition product—oxynitrohydrothymin⁴ II.

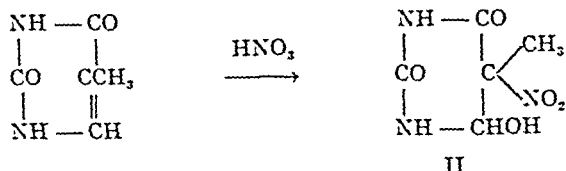


¹ *Zeitschr. f. physiol Chem.*, xlii, p. 169.

² *Ibid.*, xlviii, p. 425.

³ *Ibid.*, liii, p. 14.

⁴ Johnson: *Amer. Chem. Journ.* (To be published in vol. xl.)



In a paper, entitled "Die Constitution des Thymins,"¹ Steudel has described the action of concentrated nitric acid on thymine. He obtained a compound to which he assigned the empirical formula, $\text{C}_4\text{H}_4\text{O}_3\text{N}_4$. He gave no melting point for the compound, but states that it did not contain water of crystallization; that it was soluble in warm water and ammonia and gave, on reduction, a derivative which responded to Weidel's alloxan reaction. The data which I have obtained² seem to indicate that Steudel's *nitrothymine* was a secondary decomposition product and not a simple thymine derivative.

The formation of the hypoxanthine II, involves a direct addition of nitric acid to the double bond between the four and five positions of the pyrimidine ring. Oxynitrohypoxanthine II, exists in two modifications which I have designated by the Greek letters α and β . The two isomers are formed under practically the same conditions. The α -derivative is the stable modification and melts at 183° to 185° . The β -derivative melts at 230° to 235° and rearranges to the α -form at the ordinary temperature. These isomeric oxynitrohypoxanthines are especially characterized by their crystalline habit, and are converted quantitatively into thymine by reduction with tin and hydrochloric acid.

5-Nitrothymine and oxynitrohypoxanthine show a remarkable difference in solubility in cold, absolute ethyl alcohol. The hypoxanthine is extremely soluble in this reagent, while 5-nitrothymine requires approximately 800 to 900 parts of cold alcohol for complete solution. I have devised a simple method of separating thymine from thymine by the use of this difference in solubility in alcohol. The mixture of thymine and thymine is treated with the proper proportions of fuming nitric acid (sp. gr. 1.5) and converted into 5-nitrothymine and oxynitrohypoxanthine respectively.

¹ *Zeitschr. f. physiol. Chem.*, xxxii, p. 241.

² *Loc. cit.*

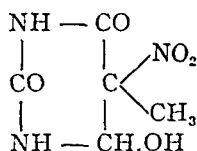
The conditions of this operation are described in detail in the experimental part of this paper. The hydrothymin is then separated from nitrouracil by trituration with absolute alcohol. The hydrothymin can be identified by its melting point and crystalline habit. Furthermore the thymin can be recovered by reduction of the hydropyrimidin with tin and hydrochloric acid. The nitrouracil is identified by elementary analysis and reduction to 5-aminouracil¹ with aluminum amalgam. This base gives a characteristic picrate which melts at 247°.²

The Action of Nitric Acid on Thymin.

Nitric Acid (sp. gr. 1.415): Five-tenths of a gram of thymin was dissolved in 2 cc. of concentrated nitric acid and the solution evaporated to dryness on the steam bath. There was no evidence of any reaction and the thymin was recovered unaltered. It deposited from hot water in plates that decomposed at about 321°. When mixed with pure thymin this decomposition point was not lowered. Analysis (Kjeldahl):

	Calculated for C ₅ H ₈ O ₂ N ₂ :	Found:
N.....	22.22	22.33

Nitric Acid (sp. gr. 1.5): *The Formation of α-Oxynitrohydrothymin:*



Thymin dissolves in cold, fuming nitric acid with slight evolution of heat. If red fumes are evolved by this treatment it is an indication that the thymin is not pure. The hydropyrimidin was obtained perfectly pure, and the yield was quantitative, when thymin was treated with fuming nitric acid under the following conditions: One gram of thymin was dissolved in 6 cc. of nitric acid and the solution allowed to evaporate at ordinary temperature. The nitropyrimidin deposited in large, well-de-

¹ Behrend and Grünwald: *Ann. d. Chem.*, cccix, p. 256.

² Wheeler and Bristol: *Amer. Chem. Journ.*, xxxiii, p. 437.

veloped prisms or blocks (see figure) which decrepitated above 130° and melted at 183° with violent effervescence. The compound was soluble in hot water, extremely soluble in alcohol, and separated from both these solvents in blocks that melted at 183° to 185° . It was insoluble in benzene. The pyrimidin did not lose weight after drying for 1.5 hours at 100° to 110° and again for one-half hour at 110° to 115° . When heated above 120° it slowly underwent decomposition. The composition of the compound was not altered by recrystallization from absolute alcohol (Analysis III):

0.2086 gram substance gave 0.2454 gram CO_2 and 0.0695 gram H_2O .
Nitrogen determinations (Kjeldahl):

	Calculated for $\text{C}_5\text{H}_7\text{O}_5\text{N}_3$:	Calculated for $\text{C}_5\text{H}_6\text{O}_2\text{N}_2$ (Thymin):	I.	Found: II.	III.
C.....	31.74	47.61	32.08		
H.....	3.70	4.76	3.70		
N.....	22.22	22.22		22.00	21.95

α -Oxynitrohydrothymin dissolves in water giving an acid reaction. The pyrimidin undergoes decomposition when its aqueous solution is boiled. The addition of barium hydroxide to its aqueous solution produces no precipitate or color. No thymin deposited when an alcoholic solution of the pyrimidin was treated with ammonia. When the nitropyrimidin was dissolved in concentrated sulphuric acid, and a few drops of ferrous sulphate solution added, the characteristic test for nitric acid was obtained.

α -Oxynitrohydrothymin can also be prepared by dissolving thymin in fuming nitric acid (1.5) and evaporating the solution to dryness, at once, on the steam bath. This method of nitration can be recommended for preparing quickly small quantities of the hydropyrimidin. The yields are not quantitative since part of the thymin undergoes oxidation. The best results are obtained by this method, when 1 gram portions of thymin are nitrated under the following conditions: One gram of thymin is dissolved in 4 cc. of nitric acid (1.5) and the solution evaporated to dryness, at 100° , as quickly as possible. The nitrothymin is obtained as a colorless, crystalline residue which crystallizes from water in blocks decomposing at 183° to 185° . It is possible, in this manner, to prepare several grams of the hydropyrimidin

in a few minutes. The yields obtained by this method of nitration were very uniform and are given in the table below:

	Nitric acid. sp. gr. 1.5	Weight of thymin.	Weight of crude oxynitrohydro- thymin.	Weight of hydropyrimidin after crystalliza- tion from water.	Percentage of theoretical.
	cc.	grams.	grams.	gram.	per cent.
1	4	1.0	1.1	0.80	57.1
2	4	1.	1.1	0.82	58.5
3	4	1.	1.1	0.79	56.4
4	4	1.	1.1	0.81	57.8
5	8	2.	2.15	1.60	55.5
6	4	1.	1.2	0.90	64.4
7	8	2.	2.1	1.50	53.5

Crystallography of Oxynitrohydrothymin by W. E. Ford.

The pyrimidin crystallizes in the triclinic system, showing a combination of b (010), c (001), a (100), M (110), d (034) and x (111). The crystals were small, averaging about 2 mm. broad by 1 mm. thick. In habit they present the appearance of diamond shaped tables with beveled edges, as is illustrated in the

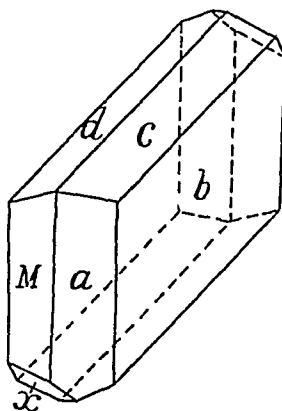


figure. The face b (010) is always the most prominent, while the prism with the pinacoid a , and the base with the dome d form the beveling faces. Frequently one of these latter faces is much subordinated in size, or may be entirely wanting. The negative pyramid x is always small and often not present. The crystal

faces, although distinct to the eye, were very poorly adapted for measurement with the reflection goniometer. They were usually quite rough or curved and gave indistinct and broad signals on the goniometer. A series of the crystals were measured on the two circle goniometer and the average of the best readings obtained were taken for the fundamental angles, but the angles as given and the crystallographic constants calculated from them can be considered only as approximate. The measured angles were as follows:

$$(010) : (110) = 63^{\circ} 54'.$$

$$(010) : (100) = 96^{\circ} 15'.$$

$$(010) : (001) = 73^{\circ} 38'.$$

$$\text{Zone } (010).(110) : \text{zone } (010).(001) = 79^{\circ} 35'.$$

$$\text{Zone } (010).(110) : \text{zone } (010).(101) = 50^{\circ} 5'.$$

$$(010) : (034) = 71^{\circ} 33'; \text{ calc.} = 71^{\circ} 46'.$$

Using the first five measurements as fundamentals the crystal constants were calculated to be:

$$a : b : c = 0.578 : 1.000 : 0.420. \alpha = 107^{\circ} 35', \beta = 100^{\circ} 25', \gamma = 80^{\circ} 59'.$$

The crystals show a good cleavage parallel to c (001). On account of the nature of the material only a few of the optical facts concerning the compound could be determined. It possesses a strong double refraction. The extinction direction on b (010) is inclined to the edge between b and a at 31° . The crystals when looked at in the polariscope in a direction perpendicular to c (001), the cleavage face, show the emergence of an optic axis nearly in the center of the field.

Reduction of α -Oxynitrohydrothymine with Aluminium-Amalgam.

Two and five-tenths grams of the hydropyrimidin were dissolved in cold water and reduced for 1.5 hours with an excess of aluminium-amalgam. The temperature was not allowed to rise above 40° during the reduction. After filtering from aluminium-amalgam and aluminium hydroxide the filtrate was evaporated to dryness. I obtained a crystalline deposit which was difficultly soluble in cold water, but separated from hot water in plates melting at 320° . When mixed with thymine the melting point

was not lowered. It dissolved in fuming nitric acid giving the original α -oxynitrohydrothymin melting at 183° to 185° .

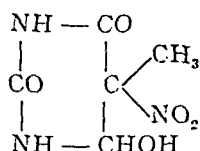
	Calculated for $C_5H_6O_2N_2$:	Found:
N	22.22	22.01

Reduction of α -Oxynitrohydrothymin with Tin and Hydrochloric Acid.

Two and two-tenths grams of the pyrimidin were reduced for one hour with an excess of tin and concentrated hydrochloric acid. The acid solution was then evaporated to dryness, the residue dissolved in water, and the tin removed by precipitation with hydrogen sulphide. When the aqueous filtrate was concentrated and cooled, thymin separated in glistening plates which decomposed at 320° . Analysis (Kjeldahl):

	Calculated for $C_5H_6O_2N_2$:	Found:
N	22.22	22.10

β -Oxynitrohydrothymin:



I have performed fifteen experiments, during this research, to determine the behavior of fuming nitric acid towards thymin under different conditions. I have taken different amounts of thymin (0.5 to 5.0 grams) and have varied the proportions of nitric acid. I have also allowed the acid solutions to evaporate under different conditions—at room temperature, on the steam oven, in a vacuum over sulphuric acid and at 100° —but in only two experiments have I observed the formation of β -oxynitrohydrothymin. The conditions, under which I obtained this isomer were as follows: One and six-tenths grams of thymin were dissolved in 8 cc. of cold, fuming nitric acid (1.5) and the solution allowed to evaporate, in the air, over night. The next morning large, transparent blocks had deposited which showed no signs of melting at 183° to 185° but decomposed at 230° to

235° according to the rate of heating. One of the crystals selected for analysis weighed 0.2600 gram. The isomer did not revert to the α -derivative when crystallized from water or absolute alcohol, but separated on cooling in well developed prisms decomposing at 230° to 236°. In another experiment 0.5 gram of thymin was dissolved in 10 cc. of fuming nitric acid and the solution allowed to evaporate over sulphuric acid, in a vacuum desiccator. I obtained practically a quantitative yield of the β -derivative melting at 230° to 235°. The compound did not lose weight when heated for one-half hour at 90° to 100°. Analyses (Kjeldahl):

	Calculated for $C_5H_7O_2N_3$:	Found:	
		I.	II.
N	22.22	22.00	22.1

Reduction of β -Oxynitrohydrothymin with Tin and Hydrochloric Acid.

This compound was reduced in the same manner as the α -derivative. The excess of tin was removed with hydrogen sulphide and the filtrate evaporated to dryness. I obtained a crystalline residue which separated from water in plates melting at 315° to 320°. The compound sublimed when heated in a test-tube and when mixed with thymin the melting point was not lowered. It dissolved in fuming nitric acid giving α -oxynitrohydrothymin melting at 183° to 185°. Analysis (Kjeldahl):

	Calculated for $C_5H_6O_2N_2$:	Found:
N	22.22	22.31

Rearrangement of β -Oxynitrohydrothymin into α -Oxynitrohydrothymin.

Some of the α -pyrimidin, melting at 183° to 185°, was preserved in a desiccator from May 29, 1907, until January 17, 1908. It apparently underwent no change and melted at 181° to 183° with effervescence. A sample of the β -pyrimidin, melting at 230° to 235° was preserved from June 19, 1907, to October 7, 1907. It then decomposed at 227° to 235°, and a nitrogen determination (Kjeldahl) gave 22 per cent nitrogen; calculated 22.22 per cent. This material was not examined again until January

16, 1908. It then had completely rearranged to the α -pyrimidin and melted at 183° to 188° with effervescence. A mixture of this material and pure α -oxynitrohydrothymin melted 183° to 186° . Analysis (Kjeldahl):

	Calculated for $C_8H_7O_5N_3$:	Found:
N.....	22.22	21.9

Method of Analysis.

The mixture of thymin and uracil, which is to be analyzed, is dried at 100° , then pulverized finely, and dissolved, at ordinary temperature (20°), in fuming nitric acid. It is essential for the success of this separation that the nitric acid have a density of 1.5 and that enough be taken to react with all the uracil and thymin present. It has been my experience that the best proportions are about 10 cc. of fuming nitric acid for 1 gram of the pyrimidin mixture. The operation is performed best in a glass, crystallizing dish of about 5.5 cm. diameter, when working with 1 gram portions.

When the pyrimidins have completely dissolved the solution is evaporated to complete dryness at a temperature of 50° to 60° . If impurities are present, which are oxidized by nitric acid at this temperature, the excess of acid can be removed by drying in a vacuum over sulphuric acid and potash. The mixture of nitrouracil and oxynitrohydrothymin is then pulverized in a mortar and triturated thoroughly with cold, absolute alcohol, using 15 cc. of alcohol for each gram of the original mixture. The insoluble nitrouracil is filtered off with suction, washed with 5 cc. of cold alcohol and purified by recrystallization from hot alcohol or water. It is identified in the manner described above.

In order to obtain the oxynitrohydrothymin the alcohol washings are allowed to evaporate spontaneously in a good draught. It is not advisable to remove the alcohol by heating on the steam bath. After removal of the alcohol the oxynitrohydrothymin is recrystallized from the least possible quantity of hot water and identified by its characteristic melting point and crystalline habit.

The results of the analyses of three different mixtures of uracil and thymin are given in the following table:

Pyrimidins.	HNO ₃ (1.5).	Nitrouracil insoluble in alcohol.	Calculated	Oxymitrohydrothymine soluble in alcohol.	Calculated	Total weight of nitrouracil and oxymitro- hydrothymine found.	Theoretical weight.
I. 0.5 uracil. 0.5 thymine.	cc. 5.0	gram. 0.61 (No test for uracil)	gram. 0.70	gram. 0.83 m. p. after first crystal- lization, 180°-182°.	gram. 0.75	gram. 1.44	gram. 1.45
II. 0.2 uracil 0.2 thymine.	2.0	0.25 (No test for uracil)	0.28	0.30 m. p. after first crystal- lization, 180°-184°.	0.30	0.55	0.58
III. 1.0 uracil 0.2 thymine	10	1.32 (No test for uracil)	1.40	0.24 m. p. after first crystal- lization, 183°. Nitrogen (Kjeldahl) = 21.9. Theoretical = 22.22.	0.30	1.56	1.70

I take pleasure in thanking Professor Ford for the crystallographic description of oxynitrohydrothymin.

COMPARATIVE STUDIES OF THE MODE OF OXIDATION OF PHENYL DERIVATIVES OF FATTY ACIDS BY THE ANIMAL ORGANISM AND BY HYDROGEN PEROXIDE.

By H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication April 13, 1908.)

- I. The catabolism of β -phenylpropionic acid and β -phenyl- β -oxypropionic acid.
- II. The oxidation of β -phenylpropionic acid, β -phenyl- β -oxypropionic acid and acetophenone with hydrogen peroxide.
- III. The fate of phenylacetic acid and of β -phenylpropionylglycocoll in the body.

Experiments upon the fate of phenyl derivatives of fatty acids in the animal body are of special interest not only on account of the fact that these substances are produced in intestinal decompositions but also because the difficultly oxidizable aromatic nucleus affords an opportunity of detecting intermediate products of metabolism which in the case of the purely fatty acids would undergo further oxidation and so escape detection. If it were possible to trace the successive steps in the oxidation of the side chain of a phenylated fatty acid the information so gained would undoubtedly throw light upon the mode of oxidation of the purely fatty acids of related structure. The present paper contains the results of such an investigation.

I. The fate of β -phenylpropionic acid in the body.

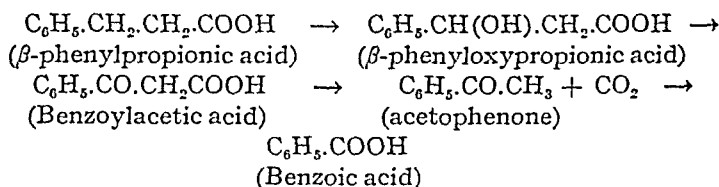
β -Phenylpropionic acid has long been known to undergo oxidation in the course of its passage through the animal body with production of benzoic acid which is excreted in the form of hippuric acid.¹



¹ E. and H. Salkowski: *Zeitschr. f. physiol. Chem.*, i, p. 169.

The formation of benzoic acid from phenylpropionic acid is now commonly considered to be the result of the direct oxidation of the hydrogen attached to the β -carbon atom in accordance with the well-known ideas of Knoop.¹ Evidence in support of this view is found in the fact that phenylacetic acid does not undergo a similar oxidation in the body, this being generally assumed to indicate that phenylacetic acid is not one of the intermediate products of the catabolism of phenylpropionic acid.

So far as I am aware, no exact picture of the mechanism of the oxidation in the body of phenylpropionic acid to benzoic acid has hitherto been put forward. The results of the present investigation indicate that the reaction takes place at least in part in accordance with the following scheme:



The evidence for this belief is based on the fact that both *β -phenyl- β -oxypropionic acid and acetophenone have been detected in the urine of dogs after subcutaneous injection of sodium β -phenylpropionate*. The β,β -phenyloxypropionic acid contains an asymmetric carbon atom and the acid found in the urine proved to be *laevorotatory*. Benzoylacetic acid was not detected but its formation must be inferred from the observed excretion of acetophenone which is readily formed from benzoylacetic acid through loss of carbon dioxide.

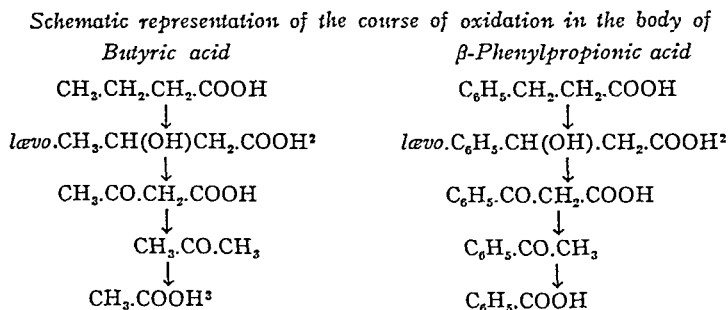
In harmony with the above hypothesis is the fact that each of the substances represented as products of intermediary catabolism of β -phenylpropionic acid (β,β -phenyloxypropionic acid, benzoylacetic acid and acetophenone) are themselves capable of further oxidation in the animal body with formation of benzoic acid.²

¹ F. Knoop: *Der Abbau aromatischer Fettsäuren im Tierkörper*, Freiburg (Baden) 1904, Ernst Kuttruff.

² Knoop: *Loc. cit.*; M. Nencki: *Journ. f. prakt. Chem.*, xviii, p. 288

Additional evidence as to the correctness of the second step in the oxidation is furnished by the detection of acetophenone in addition to hippuric acid, in the urine of dogs which had received injections of inactive sodium β,β -phenyloxypropionate. Some unchanged phenyloxypropionic acid was excreted in the urine and showed a marked laevorotation. This result is analogous to that of McKenzie's, who investigated the fate of inactive β -oxybutyric acid in the dog.¹

It will be seen from the following scheme that *there is the closest possible resemblance between the mode of oxidation in the body of phenylpropionic acid and that which is believed to represent the oxidation of butyric acid*. In each case a laevorotatory β -oxyacid is first formed, this is oxidized to a β -ketonic acid, which loses carbon dioxide passing into a ketone which then undergoes further oxidation with formation of lower acids.



These results afford the most convincing evidence of the occurrence of β -oxidation in the animal body. It is of interest to note that the excretion of β -phenyl- β -oxypropionic acid and

¹ McKenzie: *Trans. Chem. Soc.*, 1902.

² The question whether the whole of the β -oxyacids pass through the stages of ketonic acid and ketone need not be considered here. It is probable that some acetic and benzoic acids are formed by direct oxidation without passing through the stages of acetone and acetophenone, respectively.

³ Direct physiological evidence of the formation of acetic acid by the oxidation of acetone is not yet forthcoming. That acetone is to some extent oxidized appears to be demonstrated and there can be but little doubt that acetic acid is the first step in the process.

of acetophenone took place under conditions which were physiological except for the presence of the β -phenylpropionic acid in excess of the normal amount. None of the pathological conditions commonly accompanying the excretion of β -oxybutyric acid and acetone were present. It can hardly be doubted therefore that oxidation of the hydrogen attached to the β -carbon atom of saturated fatty acids constitutes the initial step in their normal catabolism.

II. The oxidation of β -phenylpropionic acid and β,β -oxyphenylpropionic acid with hydrogen peroxide.

In a number of previous communications¹ it has been shown that a surprisingly close analogy exists between the type of oxidation carried out under certain conditions with hydrogen peroxide and those occurring in the animal cell. It was therefore clearly of interest to try to imitate the reaction involved in the oxidation of β -phenylpropionic acid in the animal body, using peroxide of hydrogen as oxidizing agent. The investigation is not yet complete as the reaction is a complicated one, but the following facts have been determined.

1. Phenylpropionic acid when oxidized in the form of its ammonium salt with hydrogen peroxide yields a large amount of *acetophenone*, traces of benzaldehyde and benzoic acid and considerable amounts of aromatic oxy-acids chiefly composed of oxyphenylpropionic acids.

2. β -Phenyl- β -oxypropionic acid oxidized under similar conditions yields *acetophenone*, traces of benzaldehyde and benzoic acid.

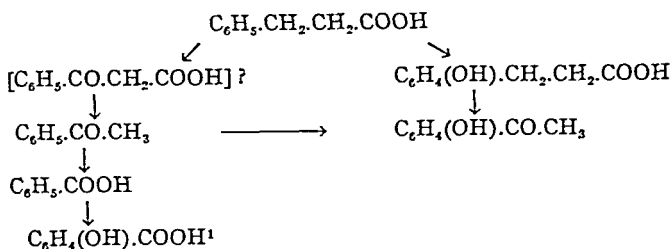
3. Acetophenone when oxidized with hydrogen peroxide in faintly ammoniacal solution yields *benzoic acid* and traces of oxybenzoic acids which result from the further oxidation of benzoic acid.² Part of the acetophenone undergoes oxidation in the nucleus, yielding oxyacetophenones.

It will be seen that the reaction is of a twofold nature in which oxidations may take place either in the side chain or in the nucleus or in both. The nuclear oxidation is similar in every

¹ This *Journal*, i, pp. 171 and 271, 1906; iv, pp. 63, 77, 91, 221, 227.

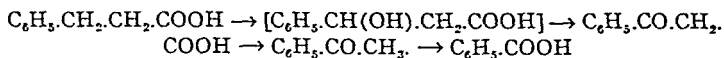
² *Ibid*, iii, p. 419, 1907.

way to that previously observed in the case of the oxidation of benzoic acid. The changes may be represented graphically as follows:



The results of the oxidations with hydrogen peroxide show that so far as concerns the side chains² the reactions have the closest resemblance to the similar oxidations in the animal body. The formation of acetophenone from phenylpropionic acid is perfectly similar to the formation of acetone from butyric acid³ and can only be explained on the basis of the assumption of the initial oxidation taking place at the β -carbon atom.

There can be no doubt but that the reaction occurs, at least in part, in accordance with the scheme already put forward as representing the course of events in the tissue oxidation although at present it has not been possible to detect the β -oxy-acid among the hydrogen peroxide oxidation products.



It is clear that the formation of acetophenone from phenylacetic acid is perfectly analogous to the formation of ketones by the oxidation of the saturated fatty acids with peroxide of hydrogen, a reaction which has proved to be general for all straight chain fatty acids up to and including stearic acid.

¹ The direct formation of oxybenzoic acids by the oxidation of oxyacetophenones has not yet been demonstrated. The oxybenzoic acids are capable of further oxidation by hydrogen peroxide. (*This Journal*, iii, p. 431, 1907.)

² At present there is no evidence that phenylpropionic acid and acetophenone undergo nuclear oxidation in the animal body.

³ *This Journal*, iv, p. 77.

III. The fate of phenylacetic acid and of phenylpropionylglycocoll in the body.

The fact that β -phenylpropionic acid undergoes oxidation in the animal body while phenylacetic acid is not oxidized but is paired with glycocoll and excreted as phenaceturic acid has generally been taken as evidence that oxidation of the hydrogen attached to the α -carbon atom of phenylated fatty acids either does not occur or takes place with difficulty. This can hardly be regarded as a fair deduction since it may be urged that the fact of the phenylacetic acid being coupled with glycocoll to form phenaceturic acid protects the phenylacetic acid from oxidation for the stability of substances of this type is well recognized. In order to test the hypothesis of the protective action of the glycocoll grouping it was decided to prepare the analogously constituted glycocoll derivative of an acid known to be capable of animal oxidation. Accordingly β -phenylpropionylglycocoll, $\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}_2\cdot\text{COOH}$, was prepared from phenylpropionyl chloride and glycocoll and was administered to dogs both by mouth and subcutaneously. It was found, however, that the glycocoll grouping did not protect the phenylpropionic acid from oxidation. No unchanged β -phenylpropionylglycocoll was recovered but much hippuric acid together with a smaller quantity of β -phenyl- β -oxypropionic acid and acetophenone. The quantity of oxy-acid appeared to be larger than would be found in the urine after administration of corresponding amounts of phenylpropionic acid but apart from this no evidence of a protective action of the glycocoll grouping was obtained.

If the phenylpropionylglycocoll had been excreted unchanged, the conclusion would have been warranted that the non-oxidizability of phenylacetic acid might be due to its protection from oxidation through coupling with glycocoll. That this was not the case does not entitle one to draw the opposite conclusion. It may be, that phenylpropionylglycocoll is hydrolyzed in the body and that the phenylpropionic acid set free then undergoes oxidation in the usual way. The experiment must therefore be regarded as indecisive but in the meantime it is safer to refrain from drawing the conclusion that α -oxidation does not occur, based on the fact that phenylacetic acid is not burned in the body. The facts so far only justify the conclusion that phenylacetylglycocoll

(phenaceturic acid) is incapable of oxidation in the animal body. It is true however that the resistance to oxidation in the organism shown by mandelic acid ($C_6H_5.CHOH.COOH$) and phenylamidoacetic acid, $C_6H_5.CHNH_2.COOH$, the oxyphenylacetic acids and other derivatives of phenylacetic acid, which are for the greater part excreted unchanged and not in combination with glycocoll, makes it very probable that α oxidation in the case of acids of this type occurs with great difficulty if at all.

EXPERIMENTAL PART.

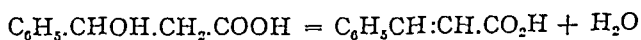
The fate of β -phenylpropionic acid in the body. A number of similar experiments were made with uniformly concordant results in which four to eight grams of the acid in the form of the sodium salt in dilute aqueous solution were injected subcutaneously into a dog weighing about fifteen kilos. In every case the urine was collected for three days following the injection. In some cases after clarifying with charcoal, it was possible to observe in the unconcentrated urine a faint laevorotation, probably due to phenyl- β -oxypropionic acid. With the exception of the substances resulting from the breakdown of the phenylpropionic acid, the presence of no abnormal urinary constituents was noted. In no case was any indication obtained of the excretion of unchanged phenylpropionic acid.

DETECTION OF ACETOPHENONE. The urines from two experiments in which 5.0 and 7.0 grams of β -phenylpropionic acid had been administered were neutralized with a little sodium carbonate solution and distilled until a few drops of the distillate gave no iodoform reaction. The distillate was then acidified with acetic acid and filtered to remove traces of fatty acids. A filtered solution of about 0.5 gram of paranitrophenylhydrazine in 40 per cent acetic acid was added to the filtrate. Precipitation of a hydrazone began at once and the separation was rendered more complete by immersing the liquid in a freezing mixture. The precipitates were then filtered off and washed with a little cold water. In one case 0.25 gram, in another 0.15 gram of crude hydrazone was obtained. It was purified by repeated crystallization from alcohol. At first the crystals melted at about 172° to 175° but after four additional recrystallizations, beautifully

formed needles melting sharply at 184° to 185° were obtained. The substance was identical in every way with acetophenone parani-trophenylhydrazone prepared for comparison from the pure ketone and corresponded exactly with the description given by Hyde.¹ The hydrazone dissolved with difficulty in dilute caustic soda with a rose color which became deep red on addition of alcohol. Part of the hydrazone was distilled with a little dilute sulphuric acid. The distillate smelt strongly of acetophenone, and gave intense positive reactions with the iodoform and the characteristic sodium nitroprusside tests.

DETECTION OF β -PHENYL- β -OXYPROPIONIC ACID AND HIPPURIC ACID. After the acetophenone had been removed by distillation, the urines were concentrated, acidified with phosphoric acid, filtered and thoroughly extracted with ether in a continuous extractor. The ethereal extract was washed with a little water, evaporated and the residue boiled with water to remove volatile fatty acids. The solution was treated with a little charcoal and on filtering and cooling an abundant yield of hippuric acid was obtained. The hippuric acid usually amounted to one-half to two-thirds of the weight of the phenylpropionic acid administered. On recrystallizing twice from hot water the hippuric acid was obtained in the form of colorless crystals, melting-point 187° .

The filtrate from the first separation of hippuric acid was evaporated to a syrup and allowed to crystallize. The residue was then stirred with a little cold water and filtered from a small additional quantity of hippuric acid. The filtrate containing the β -phenyl- β -oxypropionic acid was next examined in the polarimeter. In each of four experiments decided laevorotations varying from 1.20° to 2.05° were observed. On concentrating a crystalline residue was obtained, but it was not practicable to obtain crystals of the acid of correct melting point, owing to the presence of other substances. Accordingly the residue was boiled with dilute sulphuric acid so as to convert the oxy-acid into cinnamic acid, which could be more readily identified:



On extracting with ether a mixture of acids was obtained which was fractionally distilled from a very small flask. Some fatty

¹ *Berichte d. deutsch. chem. Gesellsch.*, xxxii, p 1814

acids came over first followed by benzoic acid derived from hippuric acid which had not been completely removed in the previous processes. When the thermometer reached 265° ,¹ the receiver was changed and a small fraction distilling between 265° and 300° was separately collected. This was crystallized twice from boiling water and yielded in one case slightly more than a tenth of a gram of pure cinnamic acid melting sharply at 133° . For further characterization the acid was converted into the dibromide by brominating in carbon disulphide solution. Platelets sparingly soluble in carbon disulphide, melting at 195° to 196° , identical with the product described by Glaser² were readily obtained. The formation of cinnamic acid from the β -phenyl- β -oxypropionic acid is exactly analogous to the production of crotonic acid from β -oxybutyric acid and takes place even more readily than the latter reaction.

The presence of β -phenyl- β -oxypropionic acid was further confirmed by the two following tests which are sensitive and serve for its detection in mixtures in which the amount of acid is too small to permit of the satisfactory isolation of the cinnamic acid.

(a) The crude acid, freed if necessary from phenylpropionic acid by steam distillation, is neutralized with ammonia and oxidized with hydrogen peroxide exactly as described on p. 428. If the oxy-acid be present acetophenone is formed and is readily identified by conversion into its nitrophenylhydrazone, by the iodoform reaction and by the nitro-prusside test. (b) The crude acid in concentrated solution is stirred in an open dish with a few drops of cold concentrated potassium permanganate solution. In the presence of β -phenyl- β -oxypropionic acid a strong odor of benzaldehyde is at once recognizable.

BENZOYLACETIC ACID. Unsuccessful attempts to isolate this acid were made. In order to test qualitatively the urine was acidified with phosphoric acid and extracted with ether and the ethereal residue taken up in dilute alcohol and tested with ferric chloride which as Baeyer has shown, gives a violet color similar to the acetoacetic acid reaction.³ The results were not sufficiently

¹ Benzoic acid boils at 249° .

² Liebig's *Ann. d. Chem.*, cxlvii, p. 91.

³ *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 2705.

decisive to warrant the conclusion of the presence of this acid. It is not improbable, however, that a very small quantity of benzoylacetic acid was present.

The fate of β -phenyl- β -oxypropionic acid in the body. β -Phenyl- β -oxypropionic acid was prepared by Fittig and Binder's¹ method by boiling β -bromhydrocinnamic acid (20 grams) with water (200 cc.) under a reflux condenser. The liquid was then cooled in ice and filtered from the precipitate of cinnamic acid containing a little adhering styrol. The filtrate was then repeatedly extracted with ether which on evaporation left 9 grams of the oxy-acid which readily crystallized. In each experiment three grams of the acid were neutralized with dilute caustic soda and injected subcutaneously into a dog weighing ten kilos. The urine was examined precisely as in the preceding case in which phenylpropionic acid had been administered. Hippuric acid amounting to about 1.0 gram was separated. Acetophenone was identified by means of its paranitrophenylhydrazone (0.09 gram) which melted at 183° to 184° after recrystallization from alcohol. Benzoylacetic acid could not be detected. Unchanged β -phenyl- β -oxypropionic acid was detected by the laevorotation (1.55°) of the aqueous solution of the ethereal extract and by the oxidation to acetophenone by hydrogen peroxide and to benzaldehyde by potassium permanganate.

The oxidation of β -phenylpropionic acid with hydrogen peroxide. The phenylpropionic acid (15.0 gram = $\frac{1}{10}$ gram molecular) was dissolved in a slight excess of ammonia and then gently warmed under a reflux condenser with 400 cc. of neutral 3 per cent hydrogen peroxide.² After a time the solution was gently boiled for three hours and then distilled. The distillate contained much ammonia and was acidified with phosphoric

¹ Liebig's *Ann. d. Chem.*, cxcv, p. 138.

² It was of interest to ascertain if the reaction could be demonstrated to occur at low temperatures as was found to be the case in the oxidation of butyric acid. (This *Journal*, iv, p. 87). Accordingly an experiment was made in which the temperature was maintained at 37° for twenty-four hours. At the end of this time the excess of hydrogen peroxide was removed by agitation with freshly precipitated silver oxide. The liquid was then acidified with phosphoric acid and distilled. The distillate readily gave qualitative reactions for acetophenone with sodium nitroprusside and with the iodoform test, but the quantity of ketone was small.

acid and redistilled. The distillate contained oil drops which proved to be acetophenone with traces of oxy-acetophenone and benzaldehyde. The acetophenone was purified by warming the distillate with ammoniacal silver nitrate in the presence of caustic soda and then acidifying and redistilling. In this way about 1.0 gram of acetophenone was obtained.¹ It readily responded to all the tests for this substance including the iodoform and nitroprusside reactions and on treatment with paranitrophenylhydrazine in acetic acid solution gave an abundant yield of the hydrazone which crystallized from alcohol in needles melting sharply at 184° to 185°. The residue from which the acetophenone had been removed by distillation was acidified with phosphoric and repeatedly extracted with rhigolene to remove unchanged β -phenylpropionic acid. In this way more than two-thirds of the phenylpropionic acid was recovered. The liquid was then extracted with ether which on evaporation left 1.5 gram of residue which crystallized on standing. It was examined with a negative result for β -phenyl- β -oxypropionic acid. The crystalline residue was fractionally crystallized, employing benzene and chloroform as solvents but it was impossible to obtain acids of constant melting point. The observed melting points varied from 80° to 120° and in every respect the substance behaved like a mixture of oxyphenylpropionic acids. The least soluble fraction was converted into the zinc salt which crystallized in small prisms and was sparingly soluble in cold water. The analysis agreed with zinc oxyphenylpropionate:

0.1065 gram salt dried at 100° gave 0.0219 gram Zn = 16.5 per cent Zn.
 $C_{18}H_{18}O_6Zn$ requires 16.52 per cent Zn.

The most soluble fraction was converted into the calcium salt which was sparingly soluble in cold water:

0.1466 gram gave 0.0401 gram $CaCO_3$ = 10.94 per cent Ca.
 $C_{18}H_{18}O_6Ca$ requires 10.81 per cent Ca.

Both fractions gave a bluish-gray precipitate with ferric chloride and all the usual reaction for phenolic acids with Millon's reagent, diazo salts, etc. It is probable that the substance repre-

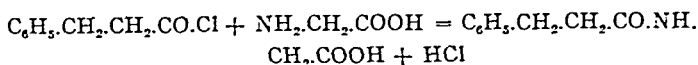
¹ Minute traces of oxy-acetophenones were present and were qualitatively detected with ferric chloride, Millon's reagent and the diazo-reaction.

sented a difficultly separable mixture of *ortho*, *meta* and *para*-oxy-phenylpropionic acids.

Oxidation of β -phenyl- β -oxypropionic acid. This acid was oxidized in exactly the same way as the β -phenylpropionic acid and the yield of acetophenone was approximately the same. As in the former case, the production of acetophenone can be demonstrated to occur even when the reaction is carried on at 37° but the change proceeds much more rapidly at higher temperatures. The acetophenone was separated by distillation and after traces of benzaldehyde had been removed by oxidizing with ammoniacal silver nitrate, it was redistilled from acid solution and identified by conversion into the paranitrophenylhydrazone which melted at 184° to 185° , after crystallization from alcohol. On acidifying the original solution from which the acetophenone had been distilled with phosphoric acid and extracting with ether a residue was obtained which contained a large quantity of unchanged β -phenyl- β -oxypropionic acid and a little benzoic acid. The latter was separated by extraction with petroleum and crystallization from water. The amount of benzoic acid was not more than 1 per cent.

Oxidation of acetophenone. Acetophenone (5 cc.) was gently warmed under a reflux condenser with 3 per cent hydrogen peroxide (100 cc.) and a few drops of ammonia. Even at the boiling point the ketone is attacked with difficulty. After several hours the solution was made decidedly alkaline with ammonia and the unchanged ketone extracted with ether. It was found that the ketone so recovered gave a strong violet red coloration with ferric chloride similar to that given by ortho-acetylphenol ($\text{CH}_3\text{CO.C}_6\text{H}_4\text{OH}$) so that it is probable that nuclear hydroxylation of the ketone had taken place to some extent as in the case of the other similar oxidations. After the unchanged ketone had been removed, the liquid was acidified with phosphoric acid and extracted with ether. The ethereal residue was small but quickly solidified and after two recrystallizations from water pure benzoic acid, melting-point 121° to 122° , was readily obtained. Minute traces of phenolic acids resulting from the further oxidation of benzoic acid could be detected in the mother liquor by means of ferric chloride and Millon's reagent, and the diazo reactions.

Synthesis of phenylpropionylglycocoll and its fate in the organism. Phenylpropionylglycocoll was prepared by the interaction of phenylpropionylchloride and glycocoll at low temperatures in the presence of excess of caustic soda.



The acid chloride was prepared in the usual way from phenylpropionic acid and phosphoric pentachloride and was fractionated *in vacuo*. Thirty grams of the acid gave 26 grams of chloride boiling at 119° to 121° under 18 mm. pressure. A number of different experiments were made to determine the best conditions for the interaction with glycocoll and the following was found most suitable.¹ Ten and eight-tenths grams of finely powdered glycocoll are added to 60 cc. of 20 per cent caustic soda solution contained in a flask which is immersed in a freezing mixture of ice and salt. Twenty-four grams of phenylpropionylchloride is then added in small portions at a time from a dropping funnel. The liquid is vigorously shaken and the temperature must be kept well below 0°. After all the chloride has been added and no oil drops are visible 7 cc. of concentrated sulphuric acid diluted with an equal volume of water are gradually added with shaking and cooling. The thick precipitate is filtered off, washed and dried and then washed again with dry alcohol-free ether, in order to remove any unchanged phenylpropionic acid. The yield of crude phenylpropionylglycocoll is practically the theoretical amount. It is purified by recrystallization from water containing a little alcohol. The substance crystallizes in long, thick needles melting at 114° to 115°. Phenylpropionylglycocoll is more readily soluble in water than hippuric acid and is almost insoluble in dry alcohol-free ether and petroleum ether, but very readily soluble in alcohol. It is readily hydrolyzed by acids with formation of phenylpropionic acid and glycocoll.

¹I am indebted to Miss Mary Dows Herter for performing these experiments.

On analysis:

0.1620 gram substance gave 0.3781 gm. CO₂ and 0.0914 gm. H₂O.
0.2018 " " " 0.0135 gm. N (Kjeldahl).

	Calculated for C ₁₁ H ₁₃ O ₃ N.	Found.
C.....	63.76 per cent.	63.65 per cent
H.....	6.28 " "	6.27 " "
N.....	6.77 " "	6.69 " "

In the first experiment 2.5 grams phenylpropionylglycocol were mixed with chopped meat and fed to a dog weighing about ten kilos. The urine was collected and concentrated, acidified with phosphoric acid and extracted with ether to which some alcohol had been added. The ethereal extract was hydrolyzed by boiling under a reflux condenser with concentrated hydrochloric acid and then distilled in steam. The distillate was extracted with ether and gave much benzoic acid but no evidence was obtained of the presence of phenylpropionic acid. The gly-cocoll group therefore had not protected the phenylpropionic acid from oxidation.

In the next experiments the phenylpropionylglycocol was administered subcutaneously as there was a possibility of the substance being hydrolyzed before absorption, if given by the mouth. Accordingly 3.0 grams of the substance were dissolved in very dilute alcohol and injected subcutaneously. The urine on distillation yielded a little acetophenone which was identified by the methods previously employed and the residue was then evaporated and extracted with alcoholic ether. The ethereal residue was hydrolyzed with sulphuric acid and then distilled in steam. The volatile acids were extracted with ether and then distilled from a very small flask. Fraction I, boiling-point up to 260° was practically pure benzoic acid. It was recrystallized from water and melted at 121° to 122°. Fraction II, 260° to 290°, was very small and contained a mixture of benzoic and cinnamic acids. Fraction III distilling over 290° weighed 0.25 gram and gave pure cinnamic acid, melting at 132° to 133°, after two recrystallizations from water. It was further identified by converting it into dibromhydrocinnamic acid, melting point 195° to 196° by brominating in carbon bisulphide solution. No indication was obtained of any unchanged β -phenylpropionic acid. β -phenyl- β -oxy-propionic acid was doubtless the mother substance of the

cinnamic acid for the former body has already been shown to occur among the oxidation products of phenylpropionic acid and the boiling with sulphuric acid for the purpose of hydrolysis would at once convert it into cinnamic acid.

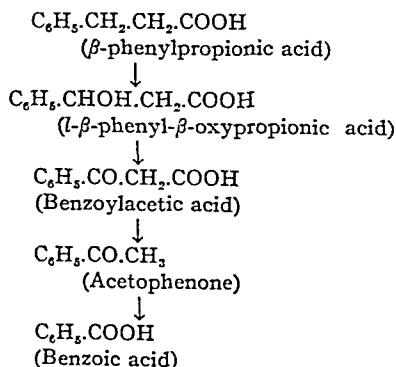
The investigation of the mode of oxidation of other phenyl derivatives of the fatty acids is being continued.

SUMMARY.

The subcutaneous injection of β -phenylpropionic acid in the form of its sodium salt in aqueous solution in amounts equivalent to about 0.3 to 0.5 gram per kilo results in the excretion in the urine of β -phenyl- β -oxypropionic acid ($\text{C}_6\text{H}_5\cdot\text{CHOH}\cdot\text{CH}_2\cdot\text{COOH}$) and acetophenone in addition to benzoic acid (hippuric acid). The β -phenyl- β -oxy-propionic acid appears to be laevorotatory and was identified by its conversion into cinnamic acid, melting point 133° , and into dibromhydrocinnamic acid, melting point 195° to 196° , and also by its oxidation to acetophenone by means of hydrogen peroxide and to benzaldehyde with potassium permanganate. The acetophenone was identified by conversion into the paranitrophenylhydrazone, melting point 183° to 184° , and by other tests.

Similar injections of sodium- β -phenyl- β -oxypropionate resulted in the excretion of acetophenone, benzoic acid (hippuric acid) and a little unchanged laevorotatory β -phenyl- β -oxypropionic acid.

The above results show that β -phenylpropionic acid undergoes oxidation in the body, at least in part, in accordance with the following scheme:



In harmony with the above scheme is the fact that each substance represented as a product of intermediary metabolism is capable of oxidation to benzoic acid in the animal body.

Benzoylacetic acid could not be satisfactorily identified in the urine but its production must be inferred from the presence of acetophenone into which it readily passes with loss of carbon dioxide. The results afford clear proof that oxidation of hydrogen attached to the β -carbon atom of fatty acids takes place in the organism and show further that there is the closest analogy between the mode of oxidation in the organism of phenylpropionic acid and that which is believed to represent the similar oxidation of butyric acid. In each case a laevorotatory β -oxy-acid is produced which then passes into a β -ketonic acid, which yields a ketone by loss of carbon dioxide. The ketone may be further oxidized to an acid.

In order to obtain chemical analogy for the type of oxidation believed to occur in the organism, β -phenylpropionic acid and β -phenyl- β -oxypropionic acid were oxidized in the form of their ammonium salts with hydrogen peroxide. The reaction takes place to a slight extent at low temperature (37°) but more readily on warming. In each case acetophenone was produced in considerable amount. The formation of this substance from β -phenylpropionic acid can only be explained on the assumption of oxidation taking place at the β -carbon atom. Acetophenone on further oxidation with hydrogen peroxide yields benzoic acid. There is therefore a close analogy between the type of oxidation believed to occur in the organism and that capable of being effected by hydrogen peroxide. In addition to the products mentioned, oxidation with hydrogen peroxide results in the formation of phenolic derivatives through oxidation in the nucleus. Oxyphenylpropionic acids, and oxybenzoic acids are produced in this way. The course of oxidation of β -phenylpropionic acid with hydrogen peroxide is represented on p. 421.

In order to test the hypothesis that the resistance of phenylacetic acid to oxidation in the organism might be due to the fact that it undergoes condensation with glycocoll and is excreted as phenyl-acetyl-glycocoll and that the glycocoll might exercise a protective action, the homologous substance, phenylpropionyl-glycocoll, was synthesized and its fate in the organism investi-

gated. No clear proof of a protective action of the glycocoll could be obtained. When the substance was given either by mouth or subcutaneously the ordinary products of the catabolism of phenylpropionic acid were formed (β -phenyl- β -oxypropionic acid, acetophenone and hippuric acid) but no unchanged phenylpropionic acid or its derivatives could be detected.

NOTE ON THE RELATIVE RATE OF ABSORPTION OF OPTICALLY ISOMERIC SUBSTANCES FROM THE INTESTINE.

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(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, April 13, 1908.)

The specific action of the living cells of the mucous membrane of the intestine in regulating absorption made it of interest to investigate the rate of absorption of such closely related substances as the dextro- and lævo-forms of lactic acid, tartaric acid, α -oxybutyric acid, β -oxybutyric acid, mandelic acid and tyrosin. As is well known, the dextro- and lævo-forms of substances have the same osmotic pressure and other physical properties with the exception of optical behavior. It is remarkable, however, that in most cases the two forms are assimilated to very varying extents by the living organism, and in accordance with this is the fact that enzymes may act on the two forms with widely varying velocities. It was clearly of interest to see whether the specific or "physiological factor" concerned in absorption from the intestine would result in the selective absorption of one form at a greater rate than the other.

The experiments were made on anæsthetized animals by taking a loop of intestine, washing with saline and then introducing a known volume (40 to 60 cc.) of a solution containing the optically inactive acid in the form of its sodium salt in slightly hypertonic solution. The loop of gut was then returned to the abdomen and partial absorption allowed to take place. After sufficient time had been allowed for the absorption of one-half to two-thirds of the original solution, the residual fluid was examined for the presence of an optically active acid. In all cases traces of proteins were removed from the solution and in some cases the residual acids were isolated in a pure state. In the case of the oxy-acids the extracts were treated with Walden's uranium reagent to increase any possible rotation. *In no case*

could any selective absorption be demonstrated. From the fact that most of the acids investigated are assimilated by the organism in a selective fashion, the conclusion appears warranted that the absorption of these substances is an independent process not involving assimilation.

PROTEIN METABOLISM IN CYSTINURIA.¹

BY CHARLES G. L. WOLF AND PHILIP A. SHAFFER.

With the Assistance of EMIL OSTERBERG and MICHAEL SOMOGYI.

(From the Departments of Chemistry and of Experimental Pathology, Cornell University Medical College, New York City.)

(Received for publication, March 18, 1908.)

This paper deals with the results of an investigation of the metabolism in two cases of cystinuria. It is unnecessary to give an outline at the beginning, either of the history or the peculiar features of the anomaly, for an analytical critique of the literature is given by Moreigne, Simon, and more recently by Neuberg in v. Noorden's *Handbuch der Pathologie des Stoffwechsels*.

One of our cases, the first to be examined, was observed over a period of eight and one-half months, during which time we have conducted five separate metabolism experiments, using different constant diets and have made complete analyses of the urine on eighty-one days. During these experiments we have administered to the patient various substances in an attempt to clear up certain specific points. As these points can be more conveniently referred to when the analytical results are presented, they will be discussed apart from the general consideration of the metabolism in this affection.

The second case is probably unique in the history of cystinuria, and presented an unusual opportunity to examine the relation

¹We wish to thank Drs. C. E. Nammack, Alexander Lambert and Cyrus Strong, the attending physicians of the Fourth Medical Division of Bellevue Hospital for their courtesy in permitting us the examination of the first patient. Our thanks are especially due to Drs. C. K. Stillman, R. R. Ryan, G. B. Emory, F. W. Rice and H. C. Thacher, house physicians in this division, for their unfailing willingness to give us every possible assistance during the experimental work. For the second case, we are indebted to Prof. L. A. Stimson and the house surgeons of the New York Hospital for great assistance during a very difficult investigation.

of bile sulphur to urine cystin in cystinuria. The patient was a woman suffering from an impaction of the ductus communis choledochus, and had been operated on for gallstones. Calculi had been removed from the gall-bladder. During the time she was under observation there was a fistulous opening, through which all the bile could be collected. Unfortunately the patient proved refractory; it was quite impossible to induce her to take a constant diet, and we were also unable to collect accurately twenty-four hour specimens of urine. It is certain that much of the urine on some of the days was passed with the stools. The case was also most remarkable in that during the course of the examination the cystinuria disappeared. While this fact has been noted by other observers, it has never occurred that the transition took place during the course of the examination. It is to our very great regret that the records of this very singular case are so incomplete.

The experiments on the two cases will be discussed separately.

The plan of our experiments was essentially the same as that adopted by Alsberg and Folin. The patient was placed on a fairly constant diet, and complete analyses of the urine made, embracing the following fractions. Total nitrogen (Kjeldahl); urea (Folin); ammonia (Folin and Boussingault-Shaffer); creatinin and creatin (Folin); uric acid (Folin-Shaffer); rest nitrogen by difference; total sulfur, inorganic sulfates, ethereal sulfur and neutral sulfur (Folin). The analytical results with the notes regarding the specific diets will be found in the tables.

CASE I. The extract from the case history is as follows:

J. S. male, age 44. Nativity, Sweden. Admitted to Bellevue Hospital, October 19, 1906.

Present History: Has used beer and whisky freely for years. Appetite always poor. Bowels move daily. Has had gonorrhea twice in the last two years.

Previous illness: Eight years ago patient was ill for four or five months. Says illness was identical with present. Denies any other illness.

Present illness: Onset two weeks ago. Swelling of both ankles, accompanied by signs of inflammation, redness and local heat. Two days later both ankles became painful. Swelling of left hand. Both knees have become somewhat painful and stiff.

Complaints: Pain, stiffness and swelling. Temperature, 100.4° F.; pulse, 120; respirations, 24.

Physical examination: Adult male, fairly well nourished. Not acutely ill. Some pain when ankles and hand are moved. Slightly anemic. Heart normal. No fibrosis of arteries. Lungs, abdomen, liver and spleen normal. Lymph nodes not enlarged. Joints, very slight swelling and tenderness.

Diagnosis: Rheumatic fever, subacute.

The points which have been investigated with this patient are the following:

- (1) The quantitative variation in the composition of the urine with diets containing varying amounts of protein.
- (2) What proportion of the food protein sulfur is excreted as cystin?
- (3) The degree of tolerance of the cystinuric for free cystin and cystein, when given by the mouth.
- (4) The degree of tolerance of the cystinuric for cystein and cystin when administered subcutaneously.
- (5) The degree of tolerance of the cystinuric for other amino-acids.
- (6) A study of the time relations of carbon, sulfur and nitrogen, and some of the fractions of nitrogen and sulfur groups after the administration of protein.

THE QUANTITATIVE COMPOSITION OF CYSTINURIA URINES.

With the exception of a single set of analyses, by one of us and Marriott, Alsberg and Folin are the only investigators who have made a fairly complete analysis of cystinuria urines, using satisfactory methods. These authors did each day during the whole of their experiments on the effect of diet upon the excretion of cystin. We also have made complete analyses each day, and have therefore a means of comparing the metabolism of our case, in so far as it is represented by analyses of the urine, with that of Alsberg and Folin, and with normal individuals on the same diets. At the foot of Tables VII and VIII are given averages, representing normal urines (for the particular diet and amount of total nitrogen), and also the averages of Alsberg and Folin from their case of cystinuria. A comparison of these averages with the averages from our own case shows the amount and character of the deviation from the normal, and from the Alsberg-Folin patient.

In the first experiment (Table VI) in which a high protein, milk-egg diet was given, the averages of our control days compare remarkably well with those obtained by Alsberg and Folin. The only important differences are our higher rest nitrogen and higher neutral sulfur, in spite of the fact that our patient was 12 kilogrammes lighter in weight. It is worthy of note that the average percentage of neutral sulfur was 10 per cent higher.

Compared with the normal averages, we find the same variations as those noted by Alsberg and Folin. The ammonia nitrogen is about half the normal and the rest nitrogen is very high. The ratio between total sulfur and total nitrogen is quite within the normal limits; but the neutral sulfur, including the cystin sulfur is more than four times greater than normal, making the relative percentage 37.0 per cent instead of the normal 7.3 per cent. The ratio between total nitrogen and neutral sulfur is 3.1 as compared with 0.6, the normal ratio for this amount of total nitrogen.

On the low protein diet, the abnormalities become much less pronounced. (See control averages, Tables VII and VIII.) When the patient catabolized only little food protein the composition of the urine was more nearly, but not quite normal. On such a diet, the ammonia is practically normal. The rest nitrogen, while still high is decidedly nearer the normal figures, in one experiment (Table VII) being 0.58 gram, and in the other (Table VIII) 0.79 gram, as compared with Alsberg and Folin's 0.69 and 0.86 gram, and the normal 0.40 and 0.42 gram.

The absolute amount of rest nitrogen in this case as in the normal depends upon the amount of protein catabolized. As an instance of this fact, the reader is referred to the results of the experiment (Table VIII) in which 50 grams of casein were administered while the patient was on a low protein diet. The rest nitrogen was increased from about 0.8 gram to 1.14 gram, the average of two days after the casein ingestion. The ratios between the sulfur and nitrogen are normal (compared with Shaffer's averages from a normal individual on the same rice-cream diet); but the ratio of neutral sulfur to total nitrogen is again high, being in one experiment 5.5 per cent as against the normal 1.6 to 2.5 per cent.

The marked anomalies in our case of cystinuria as well as in

that of Alsberg and Folin are therefore, (a) a very high neutral sulfur, (b) remarkably low ammonia nitrogen, and (c) high rest nitrogen.

The increase of neutral sulfur is certainly in part and possibly wholly due to the sulfur of the cystin. The nitrogen of the cystin is included in the "rest nitrogen" fraction, but even assuming that all the excess of neutral sulfur is due to the cystin, *that amount of cystin is not sufficient to explain the high rest nitrogen.* For instance, on the high protein diet, the excess of neutral sulphur is $(0.45 - 0.10) = 0.35$ gram, which is equivalent to 0.15 gram of cystin-nitrogen. The excess of rest nitrogen is more than 1.0 gram ($\pm 1.8 - 0.6$). *There must therefore be one or more stable nitrogenous substances outside of those examined for and other than cystin in the urine in this condition.* In the examination of the experimental sulfur anomaly produced by poisoning with brombenzol, one of us with Marriott has come to a similar conclusion. We have so far not been able to make a systematic search for these substances, and it is useless to conjecture their identity. Simon, and Alsberg and Folin suggest that diamins are present in a larger percentage of cases than is at present supposed. We have on two occasions attempted to find diamins in the urine of this patient by the Baumann-Udransky method, but without success. Moreover, diamins could not possibly be the cause of the high undetermined nitrogen, as these substances are volatile with steam, and so would be found in the urea distillate. Some experiments which we have performed show that pentamethylenediamin when added to urine does not come over in appreciable quantity either by the Folin-air method, or by the Boussingault-Shaffer method for ammonia, so that diamins would not be included in the ammonia fraction. Whatever the substances are composing the rest nitrogen in cases of cystinuria, it is evident that their amount depends upon the weight of food protein catabolized; the less the total nitrogen, the nearer to normal values does the rest nitrogen become. The low ammonia excretion is, as Alsberg and Folin suggest, probably due to the smaller amount of protein sulfur excreted in the form of sulfates.

THE EFFECT OF THE AMOUNT OF PROTEIN INGESTED ON THE
AMOUNT OF CYSTIN EXCRETED.

A fundamental problem towards the solution of which many of our experiments have been directed is the source of the cystin which is excreted in the urine. The questions which we wished to decide were:

(1) Is the urine cystin derived directly from food protein? If so, what percentage of the food protein is excreted as cystin?

(2) Is all of the urine cystin derived directly from food protein? In other words, is the urine cystin wholly exogenous?

To answer these questions demands that we be able to determine the amount of cystin excreted. So far as we are aware, there is no accurate method for the determination of cystin in the urine. The procedure usually adopted is to weigh the cystin which has been precipitated from the urine by means of acetic acid. In some cases the urine is evaporated in a vacuum before precipitation. That precipitation by acids is not satisfactory is shown by Mester's experiment, in which he obtained but 4.0 per cent of the cystin added to urine. We also have failed to obtain even approximately complete recovery of cystin by acidification with acetic acid, and other workers have met with the same difficulty. Evaporation in a vacuum doubtless increases the yield of cystin but the results are by no means quantitative. Substances which are present in the urine hold the cystin in solution. The other methods which have been employed, such as precipitation with benzoyl chlorid, α -naphthyl isocyanate, and β -naphthalin sulfochlorid, are all unsatisfactory from a quantitative point of view, for with these reagents other substances are precipitated and an accurate separation of the cystin is impossible.¹

In our experiments, the amount of cystin excreted is deduced from the amount of neutral sulfur in the urine. In such a calculation we must assume that the whole of the excess of neutral

¹ Since this work was finished, Gaskell has suggested that the precipitation by means of acetic acid be made in the presence of acetone. It is quite possible that this method may be more satisfactory than any heretofore employed. We have not had an opportunity to give it a sufficient trial to be able to speak of its accuracy.

sulfur over the amount found in normal individuals is due to cystin. Mester, and Alsberg and Folin have also made use of this assumption. That this is not necessarily correct must be admitted, for it is by no means certain that the neutral sulfur output in a cystinuric urine, exclusive of cystin is the same as that in a normal individual; or, that the sulfur containing compounds, over and above the normal neutral sulfur are exclusively cystin.

As however the accurate determination of cystin is beset with so many difficulties, one is almost forced in a long series of experiments to deduce the amount of cystin from the excess of neutral sulfur. We believe, with Alsberg and Folin, that the calculation of the amount of cystin from the neutral sulfur is more nearly satisfactory than the results of any other method now available.

The relation of diet to the elimination of cystin was investigated very early by Pletzer and Toel, who found that foods containing a low amount of nitrogen increased the cystin output. This was confirmed by Ebstein who fed his patients beans. Cantani went so far as to prescribe a meat diet in the affection as a means of decreasing the amount of cystin. Bartels on the other hand was unable to confirm these results. Mester in 1890 reinvestigated the whole matter in a more systematic manner, and fed his patient on various diets. Unfortunately for the present purpose the amounts of nitrogen excreted in the urine are not given. He did however estimate the amount of total sulfur and total sulfate-sulfur excreted in the twenty-four hours, and from this calculated the amount of neutral sulfur.

Accepting the total sulfur output as an index of the total amount of nitrogen catabolized, the various diets given by Mester do not seem to have varied very considerably in their nitrogen content. Thus, for example, on a meat diet 1.28 grams of sulfur were eliminated, while on what is termed a carbohydrate diet, 1.07 gram of sulfur were excreted. From this one may conclude that the change in the diet, in so far as the nitrogen content was concerned, was not very radical. The highest amount of neutral sulfur was excreted on a vegetable diet, the lowest on a mixed diet. One must conclude from these observations that the cystin excretion (neutral sulfur) is greatest

on a vegetarian diet. Thus Mester apparently confirmed the statements of the older investigators.

The next detailed report of the behavior of a cystinuric with different diets is given by Alsberg and Folin. These investigators used two very different diets. One contained about 19.0 grams of nitrogen as protein, and the other consisting chiefly of starch and cream contained less than 1.0 gram of nitrogen. The averages of their results, which have already been mentioned, will be found in our tables. Their results show that, with the great decrease in food protein, there was a marked decrease in neutral sulfur excretion. On the diet containing very little protein, the neutral sulfur, in spite of being greatly diminished in absolute amount, was relatively very much more prominent than when more protein was catabolized. And, assuming that the excess neutral sulfur represents cystin, they conclude that the amount of cystin depends primarily upon the amount of protein catabolized. In view of the fact that "cystin crystals were found in the urine at the end of a thirteen day period with diet containing practically no protein," and in view of the relatively high neutral sulfur from low protein diets, they further conclude that part of the urine cystin is derived directly from the tissues.

In a recent paper Thiele has taken occasion to controvert these views. Thiele's experiments on this point are not in the least satisfactory. Not only has he used the precipitation of cystin with acetic acid, with Abderhalden's modification of preliminary evaporation *in vacuo* but he has attempted to decide the question using periods of only one day for each of the two diets. As is shown in Alsberg and Folin's experiments, the decrease of neutral sulfur (and therefore, presumably cystin) is not very well marked on the first day of a low protein diet. It is quite certain that no decisions on involved questions as these can be reached with experiments of such a type.

The following averages taken from our own tables show the relation between the cystin excretion (calculated from the excess of neutral sulfur) and the amount of total nitrogen and total sulfur catabolized.

TABLE I.

Control averages from table.	Total nitrogen of urine.	Total sulfur.	Neutral sulfur.	Normal neutral sulphur.	Cystin S. (= Neut. S. - normal neut. S.).	Cystin S per cent of Total S.	100 Cystin S. Total N.
I High protein diet.....	14.63	1.24	0.45	0.10	0.35	28.2	2.4
II Low protein diet.....	3.53	0.318	0.195	0.07	0.125	39.3	3.5
III Low protein diet.....	4.15	0.378	0.199	0.07	0.129	34.1	3.1
<i>7 mo. later:</i>							
IV Fairly high protein diet.....	11.35	0.895	0.307	0.10	0.207	23.2	1.8
IV Low protein diet.....	4.81	0.43	0.26	0.07	0.19	44.1	3.9
Alsberg and Folin.....	14.84	1.17	0.328	0.10	0.228	19.5	1.5
Alsberg and Folin.....	4.62	0.344	0.192	0.07	0.122	35.4	2.6
Alsberg and Folin.....	5.19	0.388	0.226	0.07	0.156	40.2	3.0

See also Table IV, July 1, when 12 eggs were added to the diet.

Accepting the method of determining the cystin, the figures show conclusively that the amount of cystin excreted by this case, and by Alsberg and Folin's case depends, in the first instance, on the amount of sulfur catabolized. A further argument in favor of the truth of this statement is the fact that the urines from the high protein diet deposited heavy cystin sediments, while the urines from the low protein diets contained a much smaller amount of a similar sediment. According to our results, about 25 per cent of the total sulfur was excreted as cystin when a high protein diet was fed, while on a low protein diet the cystin represented 35 to 40 per cent of the total sulfur.

Since the amount of cystin excreted depends upon the amount of food protein, we agree with Alsberg and Folin that the cystin is, in part, derived directly from the food, and is therefore of exogenous origin. Because of the relative increase of the cystin sulfur with decrease of exogenous metabolism, we must also conclude with these investigators that a part of the cystin is derived from the tissue catabolism, and is therefore of endogenous origin.

Were the cystin wholly exogenous, we should expect the

per cent of total sulfur excreted as cystin to be decreased rather than increased when the products of exogenous metabolism are greatly lowered by decreasing the protein intake.

The whole problem of exogenous and endogenous metabolism, as far as the results which can be drawn from experiments in starvation and on low protein diet is in our estimation complicated by the recent considerations of Freund and his co-workers. These investigators have directed attention to the condition which probably occurs during abstention from food. Using the older observations on the secretion of the succus entericus, they have shown that a much larger amount of protein is thrown into the alimentary canal by this means than is usually presumed to be the case. This protein is probably catabolized in exactly the same way as is food protein. It therefore must be very difficult, when one speculates on a product such as endogenous cystin, to differentiate that cystin which is actually produced without the intervention of the alimentary canal from that produced by the cleavage of protein thrown into the alimentary canal by means of the succus entericus. It is for this reason, perhaps, that the variations in starvation and in low protein diet do not appear as sharply as one might be led to expect. A type of digestion and resorption is apparently proceeding during abstention from food, which is in every way similar to that produced by the assimilation of food. In fact, it would seem from Freund's point of view that it would be quite impossible to suppress any metabolic process completely which normally has as its basis the catabolism of food protein in the intestinal canal.

THE DEGREE OF TOLERANCE OF THE CYSTINURIC FOR FREE CYSTIN AND CYSTEIN WHEN GIVEN BY THE MOUTH.

The normal human organism, as has been repeatedly shown, completely oxidizes to sulfuric acid the sulfur of cystin when this substance is given by the mouth.

In an investigation of the metabolism of a cystinuric by Loewy and Neuberg in 1904 it was shown that protein cystin given by the mouth to their patient was almost quantitatively eliminated as such, and no oxidation of sulfur to sulfuric acid took place. Alsberg and Folin, on the other hand, were unable to confirm this

interesting observation on the patient which they examined, but found that cystin when given by the mouth to a cystinuric was as completely oxidized as by the normal subject. Thiele has recently confirmed this result. It is impossible to harmonize these wholly contradictory results, except on the assumption that the metabolic processes in the two cases were very different, in spite of the fact that the two individuals excreted cystin.

In the hope of throwing some light on this situation, we have determined with our case his tolerance for both cystin and cystein when given by mouth. In all, seven experiments on this point were performed. From the results of the urine analyses, one can readily determine whether the sulfur of the ingested cystin was excreted unoxidized (Loewy and Neuberg) or was oxidized in the body, and appeared in the urine as inorganic sulfates (Alsberg and Folin). The significant results of these experiments are brought together in Table II.

It is not necessary to describe the experiments at length. (The details will be found in Table II and in the complete tables at the end of this paper.) The results lead to the conclusion that cystin, prepared from hair, cystein, prepared from protein cystin, cystin isolated from the patient's urine—that cystin which he had previously failed to oxidize—when fed by the mouth, in so far as it was absorbed by the intestine, was oxidized and the sulfur was excreted in the form of sulfates. The very slight increase in neutral sulfur after the feeding of urine cystin, cystein, and the large amount of cystin (10 grams) may possibly indicate a *tendency* towards intolerance, but it is not safe to draw this conclusion from such small differences. The increase in inorganic sulfates after each cystin or cystein feeding is very marked. In this respect also, our results are in agreement with those of Alsberg and Folin, and are contradictory to those of Loewy and Neuberg.

This is certainly a very interesting situation. As has been shown in a preceding section, food protein leads in these individuals to the excretion of unoxidized cystin; but when food protein is hydrolyzed outside the body, and the isolated cystin given them, this cystin does not pass through the body as such, but appears in the urine in the form of sulfates.

<i>Low protein diet</i>	Control average + 2 for 2 day average	8.30	0.756	0.282	37.3	0.398	52.7	9.1	4.8
	Sum of 2 days after feeding 3 gm. hair cystin	7.84	1.127	0.666	59.2	0.383	34.0	14.4	4.9
	Increase following hair cystin by mouth	-0.46	-0.371	+0.384		-0.015			
	Sum of 2 days after feeding 3 gm. cystin	8.43	1.184	0.666	56.1	0.453	38.2	14.1	5.4
	Increase following cystin by mouth	+0.13	+0.428	+0.384		+0.065			
		12.53	0.973	0.597	61.4	0.304	31.4	7.8	2.4
<i>High protein diet</i>	Day preceding cystin								
	Day following 10 gm. hair cystin by mouth	12.50	2.163	1.711	79.1	0.368	17.0	17.3	2.9
	Increase following cystin by mouth	-0.03	+1.190	+1.114		+0.064			
<i>Low protein diet</i>	Control average for 2 days	9.62	0.86	0.26	30.2	0.52	60.5	8.9	5.4
	Sum of 2 days following 3.7 gm. hair-cystin by mouth	11.40	1.44	0.86	75.5	0.52	36.0	12.6	4.6
	Increase following cystin by mouth	+1.78	+0.58	+0.50		0.00			

The marked difference in the behavior of cystin as such and the sulfur containing group of the protein molecule is worthy of further close investigation. A similar state of affairs to that seen in cystinuria is observed in animals poisoned by brombenzol. While the ingestion of meat leads to a greatly increased output in the amount of neutral sulfur and undetermined nitrogen, as *p*-bromphenylmercapturic acid, the ingestion of cystin, or of the acid itself in animals under the influence of brombenzol leads to no distinct increase in neutral sulfur.

A sufficient number of experiments with the behavior of cystin in the cystinuric are now on record to show, that with the single exception of Loewy and Neuberg's case, the capacity of the cystinuric individual for catabolizing cystin when given by the mouth is nearly complete. One can therefore be fairly positive that in the digestion and absorption of protein, hydrolysis does not take place as far as cystin, but what hydrolysis does occur, is to a degree in which the cystin group is protected from oxidation.

Loewy and Neuberg with exactly the opposite results concerning the fate of ingested cystin results were able to draw a similar conclusion, and have ingeniously suggested a method which would determine to what extent hydrolysis had occurred before absorption, by determining the point at which after giving a definite hydrolytic product of protein oxidation took place. Their suggestion appears to us well worthy of experimental trial.

THE DEGREE OF TOLERANCE OF THE CYSTINURIC FOR CYSTIN AND CYSTEIN WHEN ADMINISTERED SUBCUTANEOUSLY.

Three experiments on the fate of cystein and cystin when introduced into the organism outside the alimentary canal were performed. The results are given in the following table:

TABLE III.

	Total nitrogen.	Total sulfur.	INORGANIC SULFUR.		NEUTRAL SULFUR.		Total S. 100	Total N. 100	Neutral S. 100	Total N.
			gm.	Per cent of total S.	gm.	Per cent of total S.				
<i>High protein diet...</i>	Average of 2 days before injection (November 9 and 10).....	1.00	0.594	59.4	0.338	33.8	7.5	2.5		
	± 1 gm. cystein injected subcutaneously (November 11).....	1.29	0.685	53.1	0.540	41.9	8.3	3.5		
	Increase following injection.....	0.29	0.091		0.202					
<i>Low protein diet...</i>	Day before injection: (November 28) ± 1 gm. cystein injected subcutaneously (November 29).....	0.292	0.071	24.3	0.175	60.0	9.6	5.7		
	Increase following injection.....	0.353	0.098	27.8	0.220	62.3	10.6	6.6		
		0.061	0.027		0.045					
<i>Low protein diet...</i>	Control average.....	0.318	0.089	28.0	0.195	61.0	9.0	5.5		
	× 3 for 3 day average.....	0.954	0.267	28.0	0.585	61.0	9.0	5.5		
	4 gm. cystein injected subcutaneously 3 days following injection:									
	December 10.	0.747	0.342	45.8	0.370	49.5	18.4	9.1		
	December 11.	0.353	0.082	23.2	0.250	71.0	12.5	8.9		
	December 12.	0.423	0.112	26.5	0.275	65.0	12.0	7.9		
	Sum of 3 days following injection..	1.523	0.536	35.2	0.895	58.8	13.2	7.7		
	Increase following injection.....	0.569	0.269		0.310					

On the high protein diet, about 1.0 gram of cystin dissolved in sodium carbonate was injected into the loose tissue beneath the breast. The total sulfur was increased 0.29 gram; the inorganic sulfur 0.09 gram, and the neutral sulfur was increased 0.20 gram.

Three weeks later, about 1.0 gram of cystein was dissolved in 0.85 per cent sodium chlorid, and was similarly injected. The increase in the total sulfur was very slight, but the greater part of this increase was found in the neutral sulfur.¹

The third and last experiment on this point was the injection of 4.0 grams of cystein dissolved in 0.85 per cent sodium chlorid. The injection took place beneath the scapula of the left side.² The effects of this injection seem to be shown in the three succeeding days, and for this reason the results of the three days are given in the table. The total sulfur was increased 0.57 gram, of which 0.27 gram falls on the inorganic sulfur, and 0.31 gram falls on the neutral sulfur. From these results we can merely conclude that the subcutaneous injection of cystin and cystein lead to a marked increase in the non-oxidized sulfur, and to a smaller increase in the inorganic sulfur. Our results do not prove that the increase of neutral sulfur is due to cystin, but we believe this to be the case for the following reasons. Blum injected cystin subcutaneously into normal dogs. The sulfur was oxidized to sulfuric acid. Injected rapidly into the peripheral circulation, cystin was, in part, excreted unchanged. Injected into the mesenteric veins, no cystin could be recovered from the urine. This appeared to Blum to denote that the slowing of the circulation of cystin when perfused through the liver gave time for the substance to be oxidized to sulfates, while in the greater circulation the cystin reached the kidneys rapidly,

¹ The amount of cystein given in this injection is uncertain. The cystein was partly oxidized to cystin, and the crystals formed blocked the syringe. An unknown amount of the solution was lost.

² There was almost an immediate rise in temperature of the patient, and a very decided amount of toxic disturbance, as shown by dizziness, headache, and great malaise. The patient was put to bed. Ten days later an abscess developed. It would appear that the injection of such a large amount of cystein may be associated with distinct toxic symptoms. We were unwilling to repeat the experiment.

and was there excreted. By flooding the organism extra-intestinally as was done in our case, it is more than probable that some of the cystin reached the kidney and was excreted.

THE DEGREE OF TOLERANCE OF THE CYSTINURIC FOR SULFUR-FREE AMINO-ACIDS.

An important part of the work of Loewy and Neuberg was a study of the tolerance of their patient for amino-acids not containing sulfur. The data which have accumulated seem to show that in many cases the metabolic anomaly in cystinuria does not consist solely in the excretion of cystin, but associated with the thioamino acid in the urine may be leucin, tyrosin, and possibly tryptophan. That other amino-acids than cystin might also be present would appear very probable, yet the detection of these acids has been made in the minority of cases only. In the case of Loewy and Neuberg no other amino-acids than cystin were found in the urine. Following however the oral administration of tyrosin, leucin and aspartic acid, these substances appeared almost quantitatively in the urine. In addition, the diamino-acids, arginin and lysin were not completely metabolized as they are in the normal individual, but were excreted as the diamines, putrescin and cadaverin, although the patient under ordinary conditions did not eliminate these substances. On the other hand, Alsberg and Folin did not find any lack of tolerance on the part of their patient for aspartic acid and tyrosin. The nitrogen of these substances was excreted quantitatively as urea. Simon, Garrod and Hurthley, and Thiele were likewise unable to detect any intolerance in their several patients for amino-acids.

The results of our experiments on this point are not altogether clear. We gave our patient tyrosin, asparagin and glycocoll. The amount of tyrosin given (1.0 gram) was too small to show any effect, but the urine of that day did not show any Millon reaction. On a low protein diet we gave the patient 10.0 grams of asparagin. There was a slight increase in the rest nitrogen (0.25 gram) and a considerable increase in the urea nitrogen (0.7 gram). Again, on a low protein diet we gave the patient

20.0 grams of glycocoll. The total nitrogen of the succeeding days was increased 3.33 grams ($5.64 + 5.33 + 4.80 = 18.78 - 3 \times 4.15 = 3.33$). The urea nitrogen was increased 2.35 grams ($3.65 + 3.56 + 3.18 = 10.39 - 3 \times 2.68$) and the rest nitrogen 0.73 ($1.29 + 0.92 + 0.89 = 3.10 - 3 \times 0.79$). In each of the latter two experiments, the greater part of the nitrogen (about 70 per cent) fed as amino-acids was excreted as urea. There was therefore in our case no marked intolerance for amino-acids. It is impossible to be perfectly sure as yet as to the exact fate of amino-acids especially in man, for few experiments have been made where the amino-acids have been followed by a technique such as we have used and it would appear that experiments of this kind are urgently needed to settle the tolerance of normal individuals for the different products of the hydrolysis of proteins. It appears to us that the method of judging of their excretion by means of an analysis of the urine as we have done gives a more accurate method of determining the fate of the acids than the use of reagents for the amino-acids which give at the best very incomplete precipitations. Using for comparison the results of the various investigators who have followed the fate of the amino-acids in the human organism, it would appear that the cystinuric does not differ essentially from the normal subject in his capacity for converting these substances into urea.

THE INFLUENCE OF SODIUM CHOLATE ON THE METABOLISM OF THE CYSTINURIC.

Some years ago v. Bergmann observed that in dogs with a biliary fistula, the feeding of cystin caused no increase in the biliary sulfur; but by feeding cholic acid, especially after the ingestion of cystin, there was a notable increase of sulfur in the bile. In order to ascertain whether the increase in the neutral sulfur of the urine was due to a lack of cholic acid in the organism, so that the taurin synthesis, which undoubtedly arises from the cystin complex, was inhibited, Simon and Campbell fed cholic acid to a cystinuric. No definite change was observed in the type of the sulfur metabolism. From this they concluded, that either no combination of cholic acid with cystin

takes place as Blum had suggested, or that cystin is not changed to taurin.

In controlling Simon and Campbell's result, we gave our patient, during the time in which he was on a low protein diet (December 1, 1906) 3 grams of sodium cholate in four divided doses during twelve hours. The effect of this procedure does not agree with what Alsberg obtained with cats. The total nitrogen elimination fell from 3.61 grams to 3.03 grams. No corresponding effect was noted on the excretion of total sulfur.

It will be noted that the rest nitrogen and neutral sulfur do not entirely agree. While the relative value of the latter fell decidedly, there was a slight rise in the relation of the former to the total nitrogen. This might be explained by the occurrence of oxidation of the cystin sulfur leaving the amino group intact in the form of an amino lactic acid. As will be shown in the second case which we are about to report, the administration of cystin to a cystinuric does actually increase the amount of sulfur in the bile. In order to reconcile this observation with that of v. Bergmann who found no increase in the sulfur of the bile in normal dogs after feeding cystin, it will be necessary to perform experiments on the feeding of cystin to dogs with biliary fistulas who are under the influence of brombenzol. If v. Bergmann's experiments hold good for all normal animals, and the increase in the sulfur constituents of the bile occurs in all cystinurics, one has here a fundamental difference in metabolism which has heretofore not been pointed out.

THE EXCRETION OF CREATININ AND CREATIN IN THE CYSTINURIC

The recent experiments of Folin and of Klercker have demonstrated, contrary to what has been the accepted idea, that there is no direct relationship between the creatin taken in with the food and the amount of creatinin in the urine. When pure creatin was fed, a part was indeed excreted as creatin, but the amount of creatinin was not affected. When however creatinin was fed, at least 80 per cent of this substance passed through the body unchanged. While it was *a priori* unlikely that a cystinuric would behave differently from the normal subject

with respect to these substances, the opportunity of observing a patient on a diet which would display any anomaly was not to be neglected. Accordingly, on a low protein diet (Table II), 5.0 grams of creatin (= 1.6 gram of nitrogen) were given by the mouth. In the following 20 hours, 1.44 gram (= 0.46 gram of nitrogen) were eliminated. The creatinin excretion was not affected.

Four days later, 5.0 grams of creatinin were given by the mouth. In the following twenty hours, 3.2 grams of this substance, in addition to the usual amount of creatinin which was to be found in his urine were excreted on this day. This equals an excretion of unchanged creatinin for the first twenty hours of 64 per cent. With the normal subject, Folin found 80 per cent of the creatinin administered excreted unchanged in the urine. Folin's normal subject, receiving about 1.0 gram of nitrogen in his food, excreted from 0.0 to 20 per cent of the ingested creatin and about 75 per cent of the ingested creatinin. Our cystinuric patient, on a diet containing about 5 grams of nitrogen excreted 64 per cent of the ingested creatinin and 29 per cent of the ingested creatin. Sufficient data are not at hand to determine whether or not the excretion of exogenous creatinin by this patient is normal.

THE TIME RELATIONS OF CARBON, NITROGEN AND SULFUR IN CYSTINURIA.

As one of us has stated in a previous paper in this *Journal*, the amount of work which has been done on the time relations of the individual components of the urine, outside of the carbon and nitrogen is exceedingly small. When one comes to examine the literature of pathological conditions, practically no information is available as to the effect of abnormal conditions on the time relationships of excretion. This is especially the case when one comes to deal with relationships involving hourly examination.

Our object in performing this experiment was to ascertain if any one of the anomalies which are found in cystinuria, viz: low ammonia, low urea, high undetermined nitrogen or high neutral sulfur made their appearance at any specific time after

the ingestion of protein. In the case of the last two fractions one should be able to observe whether the suspected non-sulfur amino-acid fraction made its appearance in advance of the cystin. It is very unfortunate that no experiments on normal individuals are available with which to compare this experiment. Outside of the experiments of v. Feder with dogs on hourly relations of sulfur, nitrogen and phosphorus, Tschlenoff and Slosse and Hamäläinen and Helme who fractionated the sulfur compounds of the urine we have no standards for comparison. The experiments of the Swedish investigators were unfortunately done with periods of twenty-four hours, and are therefore not comparable with ours.

The plan of the experiment was as follows: While the patient was on a non-nitrogenous, starch-cream diet (for some time), 50 grams of casein (Hammarsten) of Kahlbaum was administered in one dose at 9 a. m. Every four hours following the administration, the urine was collected separately and analyzed. Owing to the number of determinations which we wished to make, this seemed to be the most frequent collection which we could employ. The methods used for analysis were the same as those employed in the rest of the work. The urines were analyzed for carbon by evaporating a measured volume of the urine at a low temperature in a weighed dish and burning a fair sample of the weighed residue. Owing to the small quantity of urine available, the determination of uric acid was not performed. This component is included in the undetermined nitrogen.

Results. During the first four hours all the component parts of the urine were at a low level. During the second period, eight hours after the administration of the casein, there was a sudden rise in the excretion of all the constituents. Two of the components reached their maximum at this time (see Fig. 1). The remaining fractions reached a maximum at the third period. It will be seen that there is a much greater delay in the elimination of sulfur and nitrogen than was found by Feder in his experiments with dogs after a large amount of protein. On the other hand, the results are in agreement with what was found by one of us with Marriott with dogs under the influence of brombenzol. In administering a large quantity of meat to a dog under the influence of this poison, the maximum elimination of the nitrogen

did not take place till the end of the second four-hour period, although other fractions of the urine, notably the amino-acid nitrogen, and creatinin nitrogen reached a maximum during the first four hours.

It would be unwise to generalize from these two experiments, so widely different in character, but the similarity is sufficiently striking to be pointed out. The urines of the fourth period, that is to say, sixteen hours after the ingestion of the protein contained least of all the fractions examined. From that time on, there was a secondary rise. To what this secondary rise was due is difficult to say. It was not seen in the brombenzol experiment but is indicated in the curves of Feder's experiments with normal dogs. It is possible that in the course of digestion a preliminary breaking down and resorption of some of the products of hydrolysis takes place. This produces the first and highest rise in the curves of elimination. This is followed by a second apex due perhaps to a final resorption and catabolism of the more difficultly resorbable products.

What is well marked is the early elimination of ammonia nitrogen. From this it would appear that early deamidation and resorption of ammonia takes place. This is accompanied by the setting free of molecules containing a high percentage of carbon. It is to the elimination of these substances that the early carbon maximum is due. From Feder's experiments, it would appear that the sulfur is the component which is most quickly excreted. This is certainly not seen in these experiments. While the sulfur elimination rises during the first four hours, the maximum is not reached until eight hours have passed.

It is instructive also to consider the relations of some of the constituents to one another from the point of view of hourly excretion. In this way one may obtain information regarding the proportion of nitrogen and sulfur which are excreted as the various fractions. As with the absolute excretion, most of the nitrogen excreted as ammonia occurs in the first four hours. Three of the relation curves show a marked similarity. These are the relation of total sulfur to total nitrogen, rest nitrogen to total nitrogen and neutral sulfur to total sulfur. The first curve is more exaggerated than the other two, but the trend is in absolute agreement. It is possible to infer from this that the

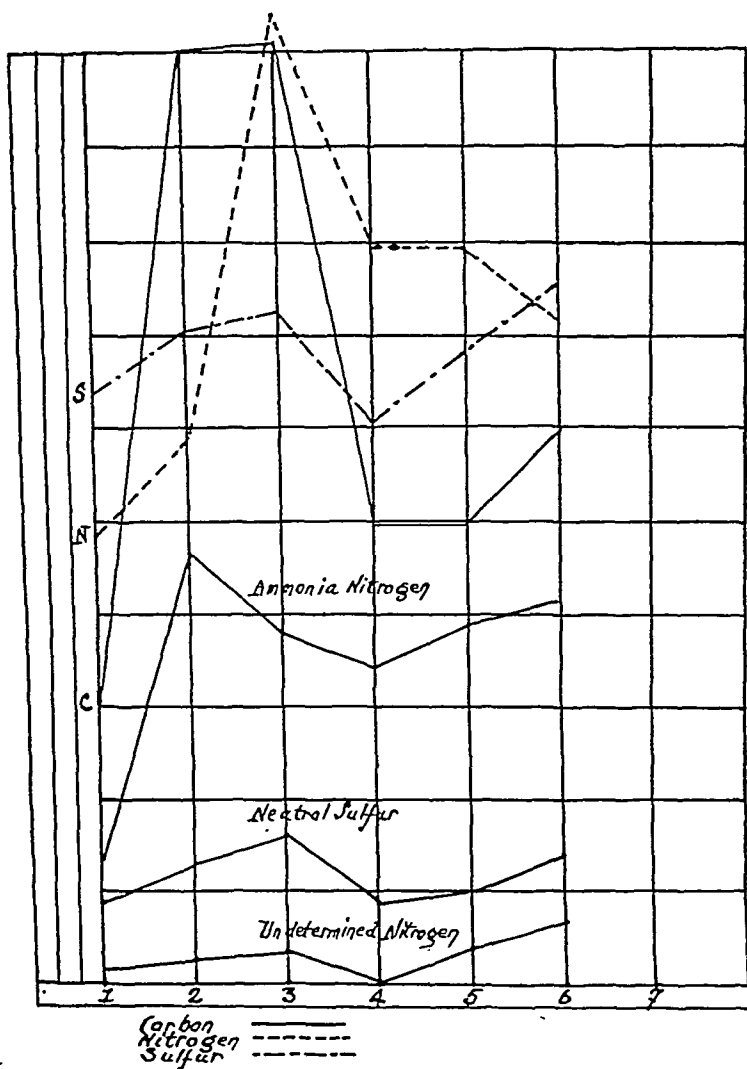


FIG. 1

TIME RELATIONS OF EXCRETION IN CYSTINURIA. (See Table VIII.)

processes giving rise to rest nitrogen and neutral sulfur are either identical or run an absolutely parallel course. Curiously enough, the course which the neutral sulfur to total sulfur takes is not quite in agreement with the three above mentioned, for instead of being the exact antipode, as might be expected, the trend of the curve is parallel to the others in the fourth period.

CASE II. This case is of such exceptional rarity, that despite the incompleteness of the results with regard to the collections of the urines and the information regarding the diet, we have decided to publish the analytical findings in full. (Table X.)

The history is as follows:

S. M. admitted to hospital September 11, 1906; discharged September 16, 1906. Readmitted November 19, discharged December 26.

Diagnosis: Typhoid fever with cholecystitis. Family history negative. Present history, none. Habits, none.

Present condition: General malaise. Physical examination: Large frame. Adipose tissue excessive. Pulse regular in size and force. Heart normal. Lungs normal. Abdomen soft, adipose tissue felt in masses. Liver percusses from fifth space to costal margin, edge not felt. A slight tenderness over the lower half of the abdomen. Spleen, percusses, enlarged. Edge felt one finger breadth below the costal margin. Skin: Few rows of spots over abdomen and chest. History omitted to September 28. At this date, jaundice of the conjunctivæ was much less. Abdomen not distended, but held rather tightly on right side. No pain or tenderness. Enlargement of the liver still persists. Note of today says cystin (?) crystals in the urine. She insists upon going home. A hard mass is felt in the right upper quadrant. It consists of rounded globules. The urinary examination showed cystin crystals September 22, 26, 28. Sp. gr. varies 1010 to 1030. The temperature varied from time of admission from 104.4° to 98.6° F. Blood examination. Leucocytes October 1, 11,000; October 4, 13,000; October 7, 10,000. Differential count gives, October 13, 13,200 leucocytes = polynuclear, 63 per cent, lymphocytes 37 per cent, hemoglobin = 58 per cent. There was no blood in the stools.

Readmission. An operation for cholecystotomy was performed November 20. The gall-bladder was evacuated of about 500 cc. of an extremely thin fluid and about 50 gallstones of various sizes scooped out. The largest about the size of a hazel nut. December 1, all drains removed, a rubber tube inserted into the opening and packed with gauze. This drainage tube was connected with a bottle. On December 5, a special diet of rice, cream, milk, crackers, butter, salt and fruit was given.

During this period, the pulse varied from 92 to 128. Respirations, 20 to 28, temperature 100° to 103°. The stools were clay colored, and free

from hydrobilirubin, except on December 5, 6, 7, 8, 9, 17, when there was a trace. On November 27, a special diet of eggs, milk and crackers was given.

The points of interest in connection with this case are:

(1) The effect of the diversion of bile on the excretion of cystin in a cystinuric.

One of us some time ago made a detailed examination of the protein metabolism in a case of complete biliary fistula. The urine was examined during periods of high and low protein diet which were free from purins. As a result of this study, it did not appear that the neutral sulfur was derived from the taurin of the bile, except in those cases associated with icterus where resorption of bile from the biliary passages took place. The neutral sulfur of the urine was not lower than is normally the case on the same quality and quantity of food. This first patient is of interest as furnishing us with standards for comparison in the present examination.

In comparing the results of the analysis of the urine and the bile of the two cases, it does not seem that outside of the effect of cystinuria itself on the urine, any marked change was to be found as a result of the diversion of the bile stream. With regard to the composition of the bile, some differences are to be observed, but they are not altogether pronounced. For a somewhat similar nitrogen and sulfur output the following table of comparison will serve to indicate the differences. The results have been chosen from the two cases as far as possible to be in accord.

At the beginning of our experiment with a biliary fistula in a cystinuric (23, 11) there are very marked differences both between the nitrogen and sulfur partitions of the cystinuria and the normal case. In the former the gross urea relation is much lower than the rest nitrogen higher; the alkaline sulfate sulfur ratio is remarkably low, and the neutral sulfur high. On comparing the ratios which were obtained towards the end of the experiment when the cystinuria phenomena had practically ceased, the ratios both for sulfur and for nitrogen are in fairly close agreement with the simple case of biliary fistula.

The excretion of nitrogen and sulfur by the bile is higher in the case of cystinuria than in the normal case, but the relations of the two components are nearly the same. The changes are

TABLE IV.
Cystinuria case with biliary fistula. (See Table X.)

URINE.												BILE.			
Date.	Vol- ume.	Total nitro- gen.	Gross urea N.	NH ₃ N	Creat- inin N.	Uric acid N.	Rest N.	Total sulfur.	Alkaline S.	Ethereal S.	Neutral S.	100 parts of 1000	Nitro- gen.	Sulfur.	100 S N.
			per cent.	per cent.	per cent.	per cent.	per cent.		per cent.	per cent.	per cent.				
Nov. 23	500	6.60	4.58 69.4	0.25 3.7	0.26 3.9	0.16 2.4	1.60 24.3	0.426	0.081 19.0	0.013 3.2	0.332 77.8	6.5			
Dec. 8	455	4.33	3.33 76.9	0.33 7.6	0.21 4.9	0.02 0.5	0.77 17.7	0.287	0.177 61.8	0.026 9.0	0.084 29.2	6.6	0.55	0.092	16.7
Dec. 12	720	6.81	5.90 86.6	0.65 9.6	0.23 3.5	0.04 0.6	0.58 8.4	0.414	0.218 52.7	0.082 19.7	0.114 27.6	6.1	0.60	0.072	12.0

Biliary fistula C. B. D. (Shaffer).

Oct. 31	740	7.56	6.59 87.2	0.77 10.2	0.26 3.4	0.14 1.9	0.57 8.5	0.42	0.26 61.2	0.08 18.1	0.09 20.7	5.5	0.225	0.035	15.5
Nov. 5	560	4.56	3.81 83.3	0.94 20.6	0.20 4.4	0.08 1.8	0.47 10.3	0.34	0.22 61.8	0.05 14.7	0.08 23.5	7.6	0.41	0.075	21.9

therefore of a quantitative nature only, and do not apparently involve any qualitative alteration.

(2) The disappearance of the cystinuria during the course of the examination.

In order to follow the disappearance of the cystinuria during the course of the examination, recourse must be had not merely to a qualitative test for the cystin as such, but to the relation of the various components of the sulfur. As many of the urines are incomplete, in order to form some idea of the amount of nitrogen which was being eliminated, it is necessary to take as a criterion for nearly complete urines, the day on which the highest amount of creatinin was eliminated. This was on October 23, on which day 0.26 gram of creatinin nitrogen were excreted. Placing all urines which have a creatinin nitrogen content between 0.18 and 0.23 as approximately complete, those days which can be relied upon to a certain extent are December 8, 9, 11, 12, 15, 18, 19, 20, 21, 23. The total nitrogen excretion varied from 3.60 to 7.23 grams. The lower amount is on the border line for urines on a diet sufficient in caloric value, but containing small amounts of protein. In judging the urines it seems wise to use as a standard the lowest values given by Folin on this diet.

In a review of the highest values for neutral sulfur and the lowest values for alkaline sulfur given by Folin it is found that the lowest relation of alkaline sulfur to total sulfur was 54.6 per cent for a normal subject, a patient however giving a value of 42.6 per cent. The highest relative value for neutral sulfur was 37.4 per cent. At the beginning of the present series of analyses we have alkaline sulfate sulfur forming but 19 per cent of the total sulfur, and the neutral sulfur 77.8 per cent. Immediately after this the ratio of neutral sulfur to total sulfur began to fall, and on December 8, two weeks after the first examination the ratios, compared with Folin's figures, had become altogether normal. In comparing the sulfur partition with that of the woman with a biliary fistula, it will be seen that at no time did the ratio of neutral sulfur to total sulfur in the latter case exceed 24 per cent.

The results obtained from an examination of the nitrogen partition are not quite so clear. While on the first day of the

examination the highest relative value for undetermined nitrogen to total nitrogen—24.3 per cent—was obtained, the value throughout the whole experiment was high, much above that found in normal individuals. There was however a distinct tendency to fall. One may suspect that while the cystin excretion decreased during the experiment a greater proportion of non-deamidated substances were still being eliminated than is normally the case. It has been shown on a number of occasions that the excretion of cystin is associated with the elimination of sulfur free amino acids. It may be that while the cystin output fell a disturbance leading to the excretion of these substances still continued.

During the course of this work we have had an opportunity to examine single twenty-four hour cases of cystinuria urines and in Table V are given the results of these analyses.¹

SUMMARY.

(1) *The anomalies in the metabolism of cystinuria.*

These consist in low ammonia, high undetermined nitrogen and high neutral sulfur. The high ammonia, as Alsberg and Folin suggest is due to the small elimination of sulfur in the form of sulfates. In some cases, as in the second, and in the case of Marriott and Wolf, high ammonia is indeed seen. In any event, the sum of ammonia plus urea nitrogen is below that found in normal individuals on the same diet. The high undetermined nitrogen is in part due to cystin, and is in part due to other amino-acids; for the ratio of amino-acid nitrogen to neutral sulfur is much above that found in normal subjects. Cystinuria is probably never a simple anomaly in which the cystin complex is the only part of the protein molecule which is affected. Owing to the difficulty of their separation, it is impossible to say what are the other fractions which are concerned in the increase in the undetermined nitrogen. The high neutral sulfur is probably due entirely to cystin. No evidence has been obtained that the

¹ For the first of these urines we are indebted to the kindness of Dr. Abrams of Providence, R. I., and for the second to Mr. Hoffmann, of Cornell Medical College,

TABLE V.
Urines of two other cases of cystinuria.

Volume Sp. gr. 1.0—	Total N. Reac- tion.	Gross urea N.	NH ₃ N.	Urea N.	Creatinin N.	Creatin N.	Uric acid N.	Rest N.	Total S 100 Tot. S Tot. N.	Tot. sul- fate S.	Alkaline S.	Ethereal S.	Neutral S.
		per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.		per cent.	per cent.	per cent.	per cent.
<i>Case I*</i>													
2100	14.8	11.6	0.15	11.4	0.57	0.0	0.210	2.46	1.135	0.758	0.663	0.096	0.377
17	alk.	78.2	1.0	77.2	3.8	0.0	1.4	16.6	7.6	67.3	58.8	8.5	33.5
<i>Case II†</i>													
1055	7.47	5.95	0.298	5.65	0.36	0.084	0.106	0.971	0.894	0.626	0.585	0.041	0.268
13	ac.	79.7	4.0	75.7	4.8	1.1	1.4	13.0	11.9	70.1	65.4	4.7	30.0

*3.62 gm. of crude β -naphthalin sulfochloride compounds obtained from the 24 hour urine.

†Faint Millon reaction in this urine.

fluctuations which are found in the neutral sulfur following changes in diet are due to any substances other than cystin. In other cases of cystinuria, viz: those in which amino-acids have been found in the urine, it would be highly important to determine whether the administration of protein increases the output of undetermined nitrogen above that which is found in normal subjects, and whether the tolerance for the free acids when given by the mouth is the same as in the normal. Were the tolerance normal for an individual free acid and the increase after protein feeding great, one would have an indication that these acids were also absorbed from the intestine in the form of higher complexes.

(2) *The origin of the cystin of the urine.*

The cystin in high protein feeding is largely of exogenous origin; but a part is probably not derived directly from food-protein. To what extent strictly endogenous processes play a part in its formation is impossible to say. With the excretion of the intestinal juice, catabolism of protein proceeds in the same way as after the ingestion of protein. The form in which the cystin sulfur is absorbed during the digestion of protein demands much further investigation, especially with improved methods for actually determining the cystin output in the urine, with methods which will leave no doubt that the increase in the neutral sulfur is actually cystin.

That the cystin sulfur of the protein molecule is not absorbed as cystin seems to be shown beyond reasonable doubt. This has been shown in our experiments in two ways. First: the ingestion of protein in the cystinuric leads to a greatly increased output of neutral sulfur and presumably of cystin in the urine. Cystin and cystein, administered by the mouth are completely catabolized to sulfates. Second: Cystin and cystein administered subcutaneously are in part oxidized, and in part excreted as neutral sulfur. One must therefore assume that the cystin group is not absorbed as such, but passes through the intestinal wall in combination with other amino acid groups as polypeptids or thioalbumoses. The hydrolysis of these compounds takes place perhaps in the liver.

(3) *The degree of tolerance of the cystinuric for cystin.*

The results which we have obtained are in substantial agreement with those of Alsberg and Folin, in showing undiminished

tolerance for cystin on the part of the cystinuric when this substance was given by the mouth. They are contradictory to the findings of Loewy and Neuberg. In each of the three sets of observations the results have been so decisive that it is necessary to formulate some hypothesis to reconcile them.

Alsberg and Folin believe that but one type of cystinuria exists. Neuberg classifies the affection into three divisions. While it seems probable that the latter arbitrary division will not be found adequate to completely cover the condition, cystinuria does in reality exist with different degrees of severity. It has been shown by us that cystinuria (Case II) may practically cease within a short time, and this has also been observed by others under less favorable conditions. Had it been possible to follow the tolerance of this patient for proteins, one would probably have found a point at which a definite amount of protein was without influence on the elimination of cystin. If this be so, it is reasonable to infer that in the severer grades of cystinuria, one may have those in which diminished tolerance for cystin or other amino-acids may be present. The fact also that diamins are found in certain cases and are assuredly not present in others leads one to believe that in this one also has perhaps a fundamental point of difference in the types of cases. It may again be emphasized that as yet no one has suggested what the point of connection of these substances in the urine has with the main anomalies of cystinuria.

(4) *The effect of subcutaneous injections of cystin and cystein on the cystinuric.*

Unfortunately we have no experiments on normal individuals with which to compare our results. The investigations of Blum on dogs and rabbits enable us to form some notion of the fate of cystin when introduced by paths other than the intestinal tract. He has shown that the rapidity of injection and whether the substance be introduced into the peripheral circulation or into a mesenteric vein determines to a large extent the course which its elimination takes. Blum attributes the occurrence of complete oxidation after mesenteric injection of cystin to the slowing of the blood current in the terminal vessels of the liver. From our results it is impossible to say whether an excessive excretion of cystin, over that which would be found in the normal

individual, as a result of the injection of cystin, took place. What is shown however is the capacity of this patient to oxidize a part of this cystin when introduced subcutaneously. It is probable in the flooding of the organism with such large quantities of cystin and cystein, part of the substances reached the liver and were there oxidized. Whether part of the oxidation took place in the muscles and other organs is impossible to decide. No experiments on the feeding of cystin to animals with an Eck fistula have so far been described. We hope to give the details of some experiments on this point in a later communication.

(5) *The fate of sulfur-free amino-acids.*

The results which we have obtained agree with those of Alsborg and Folin. Glycocoll, asparagin and tyrosin when administered by the mouth did not perceptibly increase the output of undetermined nitrogen. The nitrogen of these substances was almost quantitatively catabolized to urea.

(6) *The fate of creatinin and creatin in the cystinuric.*

The excretion of creatinin and creatin after administration by the mouth did not differ in any essential particular from what has been observed in normal individuals.

(7) *The effect of sodium cholate on the elimination of cystin.*

v. Bergmann showed in animals with a biliary fistula that the elimination of sulfur in the bile was independent of the ingestion of cystin, while sodium cholate increased the amount of sulfur in this fluid. The subsequent administration of cystin increased the output of sulfur above the amount of which was provoked by the ingestion of the cholate. It was therefore concluded that the secretion of sulfur in the bile was dependent on the reserve of cholic acid in the body. Simon gave cholic acid to a cystinuric in the hope of decreasing the amount of cystin excreted, but was unsuccessful. Our results with sodium cholate are in agreement with the last observation. No noticeable effect on the output of neutral sulfur was indicated after the treatment.

(8) *The time relations after protein feeding.*

The experiment performed was of such a nature that as yet we have no standards in the normal subject with which to compare it. It shows however that the maximum of nitrogen took place later than that of carbon, and that the former was coinci-

dent with that of sulfur. The maximum for ammonia nitrogen was early. The trends of the curves showing the elimination of neutral sulfur and undetermined nitrogen were alike.

(9) *Cystinuria and its relations to the bile.*

We have previously referred to the work of v. Bergmann and Simon who have investigated certain points in connection with cystin and the excretion of sulfur by the bile (section 6). In a comparison of a case of biliary fistula in an otherwise normal subject which has been reported by one of us (Shaffer) with a similar condition in a cystinuric, we have been unable to note any distinct difference in the composition of the bile which may be referred to the cystinuria, or in the composition of the urine which may be referred to the diversion of bile from the intestine. In all the analyses of the bile made on this case, the relation of sulfur to nitrogen varied only between the limits which were also found in the case of simple biliary fistula. One point however was observed which may distinguish the cystinuric from the normal individual: the administration of cystin by the mouth appeared to raise the amount of sulfur excreted by the bile. The relation of sulfur to nitrogen during this proceeding was altered. Control experiments with a normal subject with a biliary fistula with the administration of cystin must be made to decide whether the above mentioned behavior is characteristic for cystinuria.

(10) *The disappearance of the cystinuria during the examination.*

In their second paper on cystinuria, Loewy and Neuberg have referred to the cases of this anomaly which have ceased with lapse of time. The second case which we report shows this very plainly. While the distribution of the sulfur components of the urine returned almost to normal ratios, the nitrogen partition was still characterized by a very high undetermined nitrogen on some days. That the diversion of the bile was not responsible for this condition is deduced by the fact that in Shaffer's case the biliary fistula did not perceptibly change the ratios of the nitrogen constituents of the urine. We are inclined to believe that while the anomaly in metabolism which consists in the elimination of cystin disappeared during the time the patient was under observation, the high relative undetermined nitrogen indicates that a greater proportion of substances which come under the head of amino-acids as still being excreted. It is

difficult to estimate what connection, if any, existed between the diversion of the bile stream from the intestine and the disappearance of the cystin from the urine. As one of us (Shaffer) has found, the neutral sulfur of the urine in normal subjects probably does not have its source in the taurin of the bile. While it is unlikely that the establishment of a biliary fistula had any effect on the cessation of the cystinuria, the coincidence of the change in the metabolism appearing so soon after the operation is sufficiently striking to throw doubt on such a conclusion.

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NOTES ON FOLIN'S METHOD FOR THE SEPARATION OF THE ACETONE AND DIACETIC ACID OF THE URINE.

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Since the publication by Folin of a method for the separation of the acetone and diacetic acid of the urine¹ I have had occasion to put it to the test in the examination of a number of diabetic urines. The method is, it seems to me, fairly accurate and furnishes us with a new means of following the defective powers of oxidation of the body. It is the only method we possess of determining the relative quantities of acetone and diacetic acid excreted in the urine.

The following observations were made with the object of testing Folin's statements and the results fully corroborate his conclusions.

In order to test the efficiency of the air current to carry over the acetone, test solutions of acetone were made up in distilled water, 20 cc. of these solutions were treated with decinormal iodine solution and sodium hydrate, acidified with hydrochloric acid and directly titrated with a decinormal solution of sodium thiosulphate, using starch as an indicator. Other portions of 20 cc. of the acetone solutions were placed in a cylinder of the Folin ammonia apparatus with ten drops of a 10 per cent solution of phosphoric acid, ten grams of sodium chloride and a small amount of petroleum. This cylinder was connected by a tube containing cotton with a wide necked absorbing bottle fitted with a Folin tube. The absorbing bottle contained 200 cc. distilled water, an excess of decinormal iodine solution and strong sodium hydrate. In all of the observations the air was first drawn through an additional cylinder containing iodine and alkali to absorb any acetone which might be present in the air

¹ This *Journal*, iii, p. 177, 1907.

of the laboratory. The apparatus was attached to a Chapman's pump and a brisk current of air drawn through for twenty-five minutes. At the end of this period the stopper was removed from the absorbing bottle, its contents were acidified with hydrochloric acid and titrated in the absorbing bottle with a decinormal solution of sodium thiosulphate, with the following results. (1 cc. of decinormal iodine solution = 0.976 milligram of acetone.)

I. *Solution of acetone in distilled water.*

(a) 20 cc. acetone solution + 200 cc. H_2O = by direct titration 28.2 $\frac{N}{10}$ I = 1.376 grm. acetone per liter.

(b) 20 cc. acetone solution = by air current 28.0 $\frac{N}{10}$ I = 1.366 grm. acetone per liter.

II. *Solution of acetone in distilled water.*

(a) 20 cc. acetone solution + 200 cc. H_2O = by direct titration 7.9 $\frac{N}{10}$ I = 0.386 grm. acetone per liter.

(d) 20 cc. acetone solution = by air current 7.5 $\frac{N}{10}$ I = 0.367 grm. acetone per liter.

In experiment (b) no acetone could be detected in the original cylinder at the end of the experiment. In experiment (d) a trace of acetone was found but was not estimated.

It would appear from the above that practically all of the acetone of these varying strengths of solution are carried over in 25 minutes; the error in I was less than 1 per cent, the error in II was about 5 per cent.

To test the influence of the length of time during which the air current was continued the following experiments were made.

III. *Urine of Mrs. D. (diabetic) containing diacetic acid.*

Determination by air current.

(a) 20 cc. urine (25 minutes) = 0.980 grams acetone per liter.

(b) 20 " " (85 minutes) = 1.014 " " " "

IV. *Urine of Mr. A. (diabetic) containing diacetic acid.*

Determination by air current.

(c) 20 cc. urine (25 minutes) = 0.361 grams acetone per liter.

(d) 20 " " (90 minutes) = 0.376 " " " "

After each of the above diacetic acid could be detected in each of the original cylinders, in each case it is evident that a con-

siderable lengthening of the time during which the air current was passed led to a slight decomposition of diacetic acid.

The question has been raised as to whether at the end of twenty-five minutes the alkaline hypoiodite solution has become so changed as to be unable to further combine with acetone to form iodoform. To throw light on this question the following experiments were devised.

V. *Solution of acetone in distilled water.*

(a) 20 cc. acetone solution + 200 cc. H_2O = by direct titration $28.0 \frac{N}{10} I = 1.366$ gram acetone per liter.

(b) 20 cc. acetone solution = by air current (25 min.) $27.7 \frac{N}{10} I = 1.35$ gram acetone per liter.

(c) 20 cc. acetone solution, air current passed through for 25 minutes after which 5 cc. additional acetone solution was added to the original amount and the air current continued for 25 minutes more = 25 cc. acetone solution = $33.6 \frac{N}{10} I = 1.312$ gram acetone per liter.

25 cc. acetone solution calculated from (a) should = $35.0 \frac{N}{10} I = 1.366$ gram acetone per liter.

It would seem from the above that the absorbing solution was able to combine with acetone after twenty-five minutes but that it was somewhat less efficient than during the first part of the experiment.

VI. *Urine of Mrs. A (diabetic) containing diacetic acid.*

Determination by air current.

(d) 20 cc. urine (25 minutes) = 0.377 grams acetone per liter. At the end of 25 minutes an absorbing bottle was substituted containing a fresh solution of alkaline hypoiodite and the air current continued for 20 minutes longer.

= 0.0049 gram acetone per liter.

Total = 0.3819 " " " "

(e) 20 cc. urine (50 minutes) = 0.386 gram acetone per liter.

Here no advantage was gained by the substitution of the fresh iodine solution and the decomposition of diacetic acid was very slight.

Table showing relative amounts of acetone and diacetic acid as determined by the combined methods of Folin and Huppert-Messinger.

Date, 1907.	Quantity urine 24 hrs. cc.	Acetone gram per liter Folin's.	Acetone gram per liter Huppert- Messinger.	Diacetic acid gram per liter as ace- tone. By differ- ence.	Diacetic acid gram per liter as di- acetic acid.	GRAMS IN 24 HOURS.		Ratio of acetone to diacetic acid.
						Acetone by Folin's method.	Diacetic acid by difference.	
Mr. A.								
Dec. 20.....	1650	0.244	0.780	0.536	0.943	0.403	1.556	1 : 3.9
" 31.....	1860	0.386	1.528	1.142	2.010	0.718	3.739	1 : 5.2
1908								
Jan. 2.....	2150	0.268	0.791	0.523	0.920	0.576	1.978	1 : 3.4
" 3.....	1600	0.361	1.279	0.918	1.616	0.578	2.586	1 : 4.5
" 4.....	1660	0.351				0.583		
" 7.....	1220	0.377	1.035	0.658	1.158	0.460	1.413	1 : 3.1
" 9.....	1630	0.356	1.430	1.074	1.890	0.580	3.080	1 : 5.3
" 11.....	2160	0.142	0.766	0.624	1.098	0.307	2.372	1 : 7.7
" 13.....	2380	0.161	0.878	0.717	1.262	0.383	3.104	1 : 8.1
" 14.....	1700	0.434	1.425	0.991	1.744	0.738	2.965	1 : 4.0
" 27.....	2210	0.142	0.561	0.419	0.737	0.314	1.629	1 : 5.2
" 28.....	1640	0.234	1.056	0.822	1.447	0.384	2.373	1 : 6.2
" 30.....	2380	0.098	0.675	0.577	1.016	0.233	2.418	1 : 9.6
Mr. T.								
Dec. 18.....	2220	0.090	0.395	0.305	0.537	0.200	1.192	1 : 5.9
" 31.....	1050	0.181	0.488	0.307	0.540	0.190	0.567	1 : 2.9
1908								
Jan. 14.....	2000	0.105	0.629	0.524	0.922	0.210	1.844	1 : 8.8
Feb. 4.....	1800	0.132	0.625	0.493	0.868	0.238	1.562	1 : 6.6
Mrs. D.								
Dec. 27.....		0.298	0.956	0.658	1.158			1 : 3.9

From the above limited number of observations it would appear that for a given case the amount of acetone excreted is far more constant than the amount of diacetic.

There is no constant ratio between the amount of acetone and the amount of diacetic acid.

As the total amount of acetone and diacetic acid grows greater the increase is very largely due to an augmentation in the quantity of the diacetic acid.

ON THE QUANTITATIVE DETERMINATION OF ACETONE IN THE URINE.¹

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(Received for publication, February 21, 1908.)

Although a number of methods have been devised for the quantitative estimation of acetone in the urine, at the present time the method of Messinger,² as modified by Huppert,³ is the one most frequently employed, and is usually regarded as the most accurate one at our command.⁴

A recent paper by Folin⁵ offers the first satisfactory method for separately determining the acetone and diacetic acid of the urine. By this method, acetone and diacetic acid are estimated by the Messinger-Huppert method; acetone is separately estimated by drawing it into a suitable absorbing solution by means of a current of air. From these data the diacetic acid may be calculated by subtracting the amount of acetone obtained by means of the air current, from the acetone determined by the Messinger-Huppert method. Folin has shown that under the conditions of his method practically all of the acetone in 20 cc. of the urine is by a continuous current of air, in twenty-five minutes, transferred to the absorbing cylinder, and may there be rapidly estimated by titration. In a preceding paper, I have shown that my experiments following those of Folin corroborate the correctness of his statements.

While working with Folin's method, the thought occurred to me that if we could convert diacetic acid into acetone, both could be transferred by a current of air to the absorbing bottle,

¹ Read before the New York Pathological Society, February 19, 1908.

² Messinger: *Ber. d. chem. Ges.*, xxi, p. 1888.

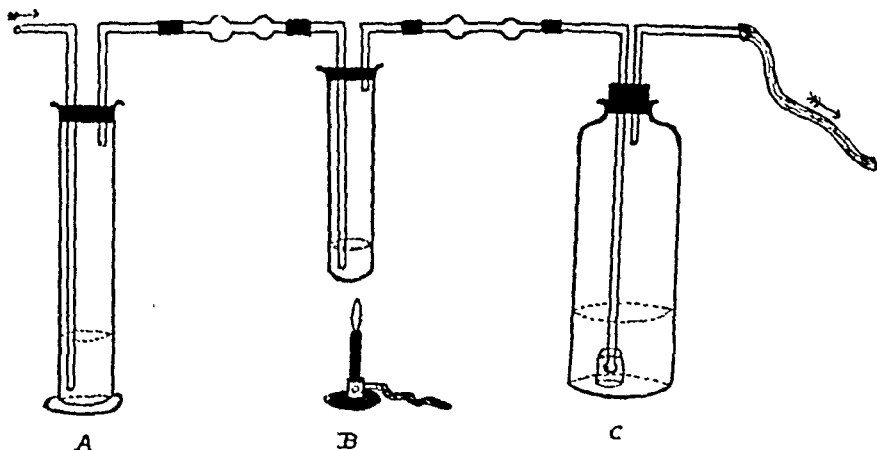
³ Huppert: *Analyse des Harns*, 1898.

⁴ Waldvogel: *Die Acetonkörper*, p. 14, 1903.

⁵ Folin: *This Journal*, iii, p. 177, 1907.

and we could thus obtain a short method for the estimation of acetone and diacetic acid in the terms of acetone. The conversion of diacetic acid into acetone and carbonic acid is readily accomplished by heating to the boiling point. The determination is conducted in the following manner:

The apparatus used is a slight modification of one devised by Folin for the estimation of ammonia.¹



A is an aerometer cylinder. B is a large test tube, two inches in diameter, raised so that it can be easily heated. C is a wide-mouthed bottle fitted with a Folin² tube. The connections between the cylinders are made with bulb tubes containing cotton. A contains alkaline hypiodite to absorb any acetone which may be present in the laboratory air. B contains 20 cc. of the urine to be examined, ten drops of a 10 per cent solution of phosphoric acid, ten grams of sodium chloride, and a little petroleum. C contains 200 cc. of distilled water, an excess of decinormal solution of iodine (carefully measured), and an excess of 40 per cent solution of sodium hydrate.

The apparatus is then connected with a Chapman pump and a steady current of air is drawn through at a moderate rate for twenty-five minutes. During this period, a lamp is placed under the tube containing the urine and the contents brought just to a boil; this is done twice, allowing an intermediate period of about five minutes.

By the application of heat the diacetic acid is converted into acetone, and at the end of twenty-five minutes will have been

¹ Folin: *Zeitschr. f. physiol. Chem.*, xxxvii, p. 161, 1902.

² Folin: *This Journal*, iii, p. 182, 1907.

drawn into the absorbing bottle and have been converted into iodoform.

The contents of the absorbing bottle (C) are now acidulated with strong hydrochloric acid, when if iodine be present in excess a brown color will develop. This is now titrated with a decinormal solution of sodium thiosulphate, using starch as an indicator as in the Messinger-Huppert method. The amount of iodine used in the formation of iodoform is calculated by subtracting the quantity of thiosulphate solution used from the quantity of decinormal iodine solution placed in the absorbing bottle. One cc. of the iodine solution is equivalent to 0.976 mgm. of acetone.

The following are a few results obtained in diabetic urines, with comparative findings by the Messinger-Huppert method.

No.	Date.	Messinger-Huppert method.	Author's method.	Difference.	Difference.
	1908.	<i>gram per liter.</i>	<i>gram per liter.</i>	<i>gram per liter.</i>	<i>per cent.</i>
1.....	Jan. 9	1.430	1.440	+0.010	+0.7
2.....	" 11	0.766	0.703	-0.063	-8.2
3.....	" 13	0.878	0.888	+0.010	+1.1
4.....	" 14	1.425	1.469	+0.044	+3.1
5.....	" 14	0.629	0.570	-0.059	-9.3

Note.—In Experiments 1, 2 and 5, after twenty-five minutes of air current and heat by the described method, a trace of acetone was found in the tube containing the urine; at times the air current was probably a little too slow in these determinations.

In three of the above determinations, the estimation was a little high, and in two the estimation was considerably too low as compared with the Messinger-Huppert method. The low determinations may be explained by the fact that the air current was too slow, as in each a trace of acetone could be detected in the original urine after the air current had passed for twenty-five minutes. The investigations of Schwarz¹ show that he was able to recover 96 to 97 per cent of the acetone of the urine by the Messinger-Huppert method.

¹ Schwarz: *Arch. f. exp. Path. u. Pharm.*, xl, 1898.

This method may be used in conjunction with Folin's method to estimate the relative amounts of acetone and diacetic acid in the urine. The apparatus and solutions are prepared as described above and an air current drawn through the urine for twenty-five minutes without the application of heat. By this means, as suggested by Folin, all the acetone is drawn into the absorbing bottle and may be estimated by titration. A fresh alkaline hypoiodite solution is then placed in the absorbing bottle; heat is applied to the urine tube to convert the diacetic acid into acetone; and this is drawn off by an air current for twenty-five minutes as above described, and is in turn estimated by titration.

The following table shows a few results obtained by this method and a comparison of these with the results obtained by the Messinger-Huppert method.

No.	Date.	ACETONE IN GRAMS PER LITER.					Difference.
		I Air current.	II Following I. Air current. and heat	III Total I and II.	IV Messinger- Huppert method.	V Difference III and IV.	
	1908.	<i>25 mins.</i>	<i>25 mins.</i>				<i>per cent.</i>
1.....	Jan. 27	0.142	0.390	0.532	0.561	-0.029	-5.4
2.....	" 28	0.234	0.781	1.015	1.056	-0.041	-3.4
3.....	" 30	0.098	0.561	0.659	0.675	-0.016	-2.4
4.....	Feb. 4	0.132	0.473	0.605	0.625	-0.020	-3.2

Note.—No. 1. Air current was very slow; at the end of the determination acetone could be detected in the urine. Nos. 2 and 3. No acetone could be detected at the end of the determinations. No. 4. A trace of acetone detected after determination.

The method as proposed affords values approximating very closely to those of the Messinger-Huppert procedure, while the time required for a determination is only half an hour as against two to two and one-half hours demanded by the Messinger-Huppert process.

Note.—To obtain a rough idea of the amount of decinormal iodine solution needed in the absorbing bottle, mix in a test tube 10 cc. of the urine to be examined and 1 cc. of a 100 per cent solution of ferric chloride. Allow this to stand for two minutes and compare the color thus developed with the color of 100 per cent ferric chloride solution in a test tube of

equal size. If the color of the two test tubes be approximately of the same depth, 10 cc. decinormal iodine solution will be sufficient to combine with the acetone set free from 20 cc. of urine. If the color of the urine mixture is darker than that of the ferric chloride solution, dilute the urine mixture with distilled water until its color approximates in depth the color of the ferric chloride solution. The amount of decinormal iodine needed can then be roughly estimated as follows:

		Fe ₂ Cl ₆ (100 per cent solution.)		H ₂ O					
Urine									
10 cc	+	1 cc.			=	needed	10 cc.	decinormal iodine solution.	
10 "	+	1 "	+	10 cc.	=	"	20 "	"	"
10 "	+	1 "	+	20 "	=	"	35 "	"	"
10 "	+	1 "	+	30 "	=	"	50 "	"	"

A CHEMICAL STUDY OF NONSTRIATED MAMMALIAN MUSCLE.

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(Received for publication, April 7, 1908.)

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Although the physiological behavior of the nonstriated, or involuntary muscles has been the subject of numerous investigations during recent years, there remains a striking paucity of data regarding the chemical characteristics of this tissue. One need only compare the statements in the older text-books of physiological chemistry with the recent summary by Grützner¹ in the *Ergebnisse der Physiologie* to see how slight have been the accessions to our knowledge. Aside from the newer contributions regarding the proteins of nonstriated muscle,² no systematic chemical studies have been made, so far as we are aware. The present paper is intended to furnish briefly the chief results of an extended analytical investigation of the stomach and urinary bladder of the pig as types of nonstriated muscular tissue masses. The

¹ Grützner: *Ergebnisse der Physiologie*, iii (2), p. 70, 1904.

² Cf. Velichi: *Zentralblatt für Physiologie*, xii, p. 351, 1899; Vincent and Lewis: *Journal of Physiology*, xxvi, p. 445, 1900-01; Vincent: *Zeitschrift für physiologische Chemie*, xxxiv, p. 417, 1902-02; Bottazzi and Cappelli: *Jahresbericht für Thierchemie*, xxxi, p. 563, 1901.

materials were prepared by careful dissection as soon as possible after removal of the organs from the slaughter house and after superficial cleaning. The fat-containing membranes and, in the case of the stomach, the mucous coats were separated before the tissue was minced and subjected to further procedures. The impossibility of obtaining "pure" specimens of muscle need scarcely be emphasized. It is hoped, however, that with the care employed the analytical data have a comparative value, enhanced by the agreement of two groups of muscles from different regions and with distinctly unlike structural environment. Connective tissues together with residual portions of blood and lymph furnish the contaminating factors.

General composition. Nonstriated muscular tissue is somewhat richer in water than the striated variety from the same species. This fact has been determined in the case of the cow's muscular uterus (containing 77 per cent of water) by Bottazzi and Cappelli,¹ the retractor penis muscle of the dog (79 per cent) and the crop of hens (78 per cent) by Panella.² The lower figures (averaging 75 to 76 per cent) usually given for adult mammalian skeletal muscle may be accounted for by the larger fat-content of the latter; flesh rich in fat is invariably relatively deficient in water. For this reason the muscle of the invertebrates is richer in water.³ A summary of the averages of our analyses follows:⁴

COMPOSITION OF NONSTRIATED MUSCLE.

	Stomach.	Bladder.
Water.....	81.1 per cent	80.1 per cent.
Solids.....	18.9 " "	19.9 " "
Ether extract.....	1.2 " "	3.5 " "
Nitrogen.....	2.8 " "	3.0 " "
Ash.....	0.9 " "	0.8 " "

¹ Bottazzi and Cappelli: *Jahresbericht für Thierchemie*, xxxi, p. 564, 1901.

² Panella: *Jahresbericht für Thierchemie*, xxxiv, p. 565, 1904.

³ For example, the adductor muscle of *Pecten irradians* contains about 80 per cent of water. Chittenden: *American Journal of Science*, x (3), p. 26, 1875.

⁴ Solids and water were determined by drying to constant weight at 110° C. The ether extract was estimated by extracting the dry tissue in a Soxhlet apparatus during twenty hours, grinding the tissue and

Glycogen. The current statement that glycogen occurs in nonstriated muscular tissue appears to have been based largely upon histological evidence.¹ We have failed to obtain evidence of more than traces, after analyzing both stomach and bladder muscle in quantities as large as 250 grams, by Pflüger's method.

Lactic acid. The evidence for the *post mortem* finding of lactic acid in nonstriated muscular tissue is conflicting.² In comparing the metabolic activities of this contractile tissue with related types it seems important to determine definitely what lactic acid, if any, is formed. The significance of the substance for the muscle has been put in a new light by the work of Fletcher and Hopkins,³ according to whom lactic acid is a product of activity and rigor under anaërobic conditions. Henze⁴ and Mendel and Bradley⁵ have found *fermentation lactic acid* alone in the muscle extractives of Octopus and Sycotypus. From both bladder and stomach muscle of the pig *we have isolated paralactic acid* and identified it as its zinc salt. The operations were conducted in one trial in the way described by Henze,⁶ the ether extractions being carried out in a Kutscher-Steudel extraction apparatus; in the other cases the familiar alcohol and ether extraction processes were employed.

2350 grams of stomach muscle (from ten pigs) yielded 1.79 gm. Zn ($C_2H_3O_2$)₂·2H₂O, or 0.05 *per cent* of lactic acid in the fresh muscle; in a second experiment, 0.05 *per cent* was found.

1314 grams of bladder muscle (from forty-nine pigs) yielded 1.47 gm. Zn ($C_2H_3O_2$)₂·2H₂O, or 0.07 *per cent* of lactic acid.

renewing the extraction another twelve hours. According to Kumagawa and Suto: *Biochemische Zeitschrift*, viii, p. 212, 1908, this method fails to remove the fat completely. Nitrogen was estimated by the Kjeldahl-Gunning process.

¹ Cf. Barfurth: *Archiv für mikroskopische Anatomie*, xxv, p. 288, 1885. In his well known paper on Glycogen, Brücke wrote: "Ich habe es in der frischen Muskelhaut eines Schweinsmagens nachweisen können, die ich in verdünnter Kalilauge zerkochte." (*Sitzungsberichte der Akademie der Wissenschaften*, Wien, 1871, lxiii, II, p. 220.)

² Cf. Nasse: Hermann's *Handbuch der Physiologie*, i (1), p. 340, 1879.

³ Fletcher and Hopkins: *Journal of Physiology*, xxxv, p. 247, 1907.

⁴ Henze: *Zeitschrift für physiologische Chemie*, xliii, p. 477, 1904.

⁵ Mendel and Bradley: *American Journal of Physiology*, xvii, p. 169 1906.

⁶ Henze: *loc. cit.*, p. 486.

ANALYSES OF ZINC PARALACTATE.

	From stomach.	From bladder.	Calculated for Zn (C ₃ H ₅ O ₂) ₂ · 2 H ₂ O:
H ₂ O.....	13.0	13.1	12.9 per cent.
Zn.....	26.9	26.8	26.7 " "

Fletcher and Hopkins¹ have given proof in the case of frog's skeletal muscle that "the survival processes in excised unstimulated muscle lead from the moment of excision onwards to a steady accumulation of lactic acid, which, under most conditions, ceases entirely with loss of irritability. * * * Partial disintegration of the muscle represents a strong stimulus, inducing this acceleration to a marked degree." After special precautions to secure a minimum of manipulation or cutting, with low temperature, etc., Fletcher and Hopkins obtained lactic acid figures for striated muscles as low as 0.02 per cent. This figure is increased ten or twenty fold after traumatic injury, such as cutting and mincing. The comparatively low figures (0.05 per cent) obtained with the nonstriated muscles without any of these special precautions to avoid the survival accumulation of lactic acid are of interest in relation to the question of rigor occurrence in such muscles.

Creatin and creatinin. There is no satisfactory evidence that creatin or creatinin occurs in the muscles of the invertebrates.² For the somewhat related nonstriated muscles of vertebrates a few older positive statements are on record. We have applied Folin's colorimetric method in precisely the way employed by van Hoogenhuyze and Verploegh³ in the study of skeletal muscles, except that a Duboscq colorimeter was used. Our data are summarized together with those on pig's striated muscle recorded by the Dutch investigators. The latter determined the total content of creatinin after boiling the extracts with acid to convert unchanged creatin into creatinin.

¹ Fletcher and Hopkins: *loc. cit.*, p. 297.

² Cf. Von Fürth: *Vergleichende chemische Physiologie der niederen Tiere*, p. 436, 1903; Mellanby: *Journal of Physiology*, xxxvi, p. 447, 1908.

³ Van Hoogenhuyze and Verploegh: *Zeitschrift für physiologische Chemie*, xlvii, p. 432, 1905.

SUMMARY OF AVERAGE RESULTS.

	Total creatinin after boiling with acid. Per cent.	Preformed creatinin. Per cent.
Skeletal muscle.....	0.39	"
Stomach "	0.079-0.093	0.026-0.052
Bladder "	0.078	0.024

The lower figures obtained for the nonstriated variety cannot be explained on the basis of a larger water content; neither does it seem probable that the larger admixture of connective tissue can account entirely for the markedly lower content of these extractives and of lactic acid. A low content of creatinin has been described in the muscles of young animals¹ and in the embryonic muscle of the pig.²

Purin bases. As in the case of striated muscles, the conspicuous purin base occurring free in the nonstriated muscles (i. e., obtainable in muscle extracts) is *hypoxanthin*.³ This was isolated and identified by analysis. The purins were separated from the water extracts of quantities of the nonstriated muscle varying from one to four kilograms⁴ by the Krüger-Schmid method after removal of coagulable protein and salts precipitable with lead acetate. Steudel's⁵ directions were also successfully followed. In two cases typical crystals of *guanin hydrochloride* were obtained in amounts too small for analysis. In one instance a trace of impure *adenin picrate* (m. p. 279° C.) was separated from stomach muscle extract. *Xanthin* could not be obtained. *Hypoxanthin* was isolated as the nitrate and analyzed:

	Calculated as $C_5H_4N_4O.HNO_3 + H_2O$:	Found:
H ₂ O.....	8.3 per cent.	8.5 per cent.
N.....	32.3 " "	32.7 " "

¹ Cf. Dorner: *Zeitschrift für physiologische Chemie*, lii, p. 264, 1907.

² Cf. Mendel and Leavenworth: *American Journal of Physiology*, xxi, p. 101, 1908.

³ Cf. also, for embryonic pig muscles, Mendel and Leavenworth, *American Journal of Physiology*, xxi, p. 102, 1908. Professor Mendel informs me that he has found hypoxanthin to be relatively conspicuous in amount in the muscular tissue of various lower vertebrates and invertebrates.

⁴ It is desirable to make the extracts with cold water as far as possible, to avoid the formation of gelatin from the connective tissue which interferes with the subsequent separations.

⁵ Steudel: *Zeitschrift für physiologische Chemie*, xlii, p. 165, 1904.

These observations are in accord with Kossel's statements that adenin and guanin occur in meat extract in traces at most.¹

Hæmoglobin. Thirty grams of fresh muscle were extracted with water, the extract strained and made up to 100 cc. Hæmoglobin was estimated colorimetrically in a Duboscq colorimeter by comparison with a standard solution containing 1 cc. of pig's defibrinated blood per liter. Lehmann² has made comparable estimations on the striated muscle of the pig.

SUMMARY.

(cc. of blood in 100 grams of muscle.)		
Heart.....	2.3	(Lehmann)
Biceps.....	1.6	(")
Loin.....	1.0	(")
Stomach.....	0.28	(Saiki)
Bladder.....	0.13-0.17	(")

If the hæmoglobin content of the blood is calculated at 13 per cent, the amount present in these nonstriated muscles would not exceed 0.03 per cent. A large admixture of blood in the muscles analyzed is thus rendered improbable.

Connective tissue. In order to compare the nonstriated muscles with the skeletal varieties in respect to content of connective tissue, estimation were made on the dissected stomach and bladder muscles as was done on meat by Schepilewsky.³ The results are tabulated below, the figures for beef being taken from Schepilewsky's paper.

	Connective tissue in fresh muscle. Per cent.
Stomach (pig).....	1.5-2.9
Bladder "	3.0-3.2
Gastrocnemius (beef).....	0.6
Gluteus "	0.5
Filet "	0.2

Inorganic salts. The important rôle of the inorganic salts or their ions in the irritability and contractile responses of muscles

¹ Kossel: *Zeitschrift für physiologische Chemie*, x, p. 263, 1885.

² Lehmann: *Zeitschrift für Biologie*, xlv, p. 335, 1904.

³ Schepilewsky: *Archiv für Hygiene*, xxxiv, p. 348, 1899, also Mendel and Goodman: *American Journal of Physiology*, iv, p. 260, 1900-01.

makes a study of these constituents of particular value. An investigation of the salts of nonstriated muscle seemed the more called for because of the fundamental differences in the physiological responses of this type of tissue. Katz¹ has enriched the literature with numerous systematic analyses of the inorganic constituents of skeletal muscle. We have duplicated these studies on the nonstriated muscles of the pig, thus supplying data which have never been furnished heretofore. The methods of analysis were precisely like those of Katz so that our data are strictly comparable with his. The results are summarized in tabular form² (see p. 490 fig.). The percentages have been calculated in several ways to permit ready comparison with other reports.

An inspection of these analytical data at once reveals several features of difference between the striated and nonstriated muscles examined. The content of sodium, iron, calcium and chlorine is distinctly higher in the dry and fat-free nonstriated tissue than pertains in the skeletal muscles analyzed by Katz. For the other elements the condition is reversed. Most striking, however, is the reversal of the relationship between sodium and potassium, and magnesium and calcium, respectively, in the two types studied. Whereas in the striated muscles potassium is more abundant (in percentage amounts) than sodium, and magnesium than calcium, this appears not to be true for the nonstriated tissue examined.³ Such variations in the interrelation of sodium and potassium have long ago been pointed out.⁴

¹ Katz: *Archiv für die gesamte Physiologie*, lxiii, p. 1, 1896; estimations of Fe in muscle are also given by Schmey: *Zeitschrift für physiologische Chemie*, xxxix, p. 215, 1903.

² It seems unnecessary to extend the limits of this paper by presenting the analytical data and protocols in detail here. Two larger samples were carefully prepared from each type of muscle by drying and extraction with ether. The results are calculated for the fresh material (fresh muscle) and the dried fat-free muscle (dried muscle). Complete details are recorded in the author's Dissertation, Yale University, 1907.

³ In the flesh of marine animals alone Katz found Ca to preponderate similarly.

⁴ Cf. Kühne: *Lehrbuch der physiologischen Chemie*, p. 333, 1866. In the chapter on "Die glatten Muskelfasern," he says: "Die Aschenbestandteile wurden bis jetzt sehr abweichend von denen der quergestreiften Muskeln gefunden, nämlich immer reicher an Natron als an Kali."

TABLE I.
Composition of the "dried" muscle tissue (parts in 100 grams).

Source.	K.	Na.	Fe.	Ca.	Mg.	Cl.	S.	P.	K ₂ O.	Na ₂ O.	Fe ₂ O ₃ .	CaO.	MnO.	Cl ^{as} NaCl.	SO ₃ .	P ₂ O ₅ .
Stomach I.....	0.415	1.549	0.028	0.111	0.026	0.922	0.444	0.381	0.500	2.085	0.040	0.155	0.045	1.521	1.110	0.857
" II.....	0.332	1.095	0.036	0.117	0.023	0.808	0.445	0.428	0.400	1.475	0.052	0.164	0.038	1.333	1.111	0.974
Bladder I.....	0.342	1.058	0.042	0.205	0.013	0.816	0.424	0.416	0.412	1.426	0.060	0.287	0.022	1.346	1.060	0.955
" II.....	0.204	1.045	0.049	0.187	0.012	0.786	0.373	0.437	0.245	1.408	0.070	0.262	0.020	1.297	0.933	1.000
Nonstriated muscle (average).....	0.323	1.187	0.039	0.155	0.019	0.833	0.422	0.416	0.389	1.599	0.056	0.217	0.031	1.374	1.054	0.947
Striated muscle (Katz's average).....	0.936	0.575	0.022	0.030	0.104	0.179	0.754	0.735	1.128	0.775	0.031	0.042	0.173	0.454	1.882	1.796

TABLE II.
Composition of the "fresh" muscle tissue (parts in 100 grams).

Source.	H ₂ O.	K.	Na.	Fe.	Ca.	Mg.	Cl.	S.	P.	K ₂ O.	NH ₄ O.	Fe ₂ O ₃ .	CuO.	MgO.	Cl as NaCl.	SO ₃ .	P ₂ O ₅ .
Stomach I.....	81.4	0.081	0.302	0.005	0.022	0.005	0.180	0.087	0.072	0.098	0.407	0.008	0.030	0.009	0.297	0.216	0.162
" II.....	80.8	0.077	0.255	0.008	0.027	0.005	0.188	0.101	0.081	0.093	0.344	0.012	0.038	0.009	0.311	0.259	0.184
Bladder I.....	80.6	0.070	0.216	0.009	0.042	0.003	0.166	0.086	0.083	0.084	0.291	0.012	0.059	0.005	0.275	0.216	0.190
" II.....	79.6	0.039	0.200	0.009	0.032	0.002	0.150	0.071	0.087	0.047	0.269	0.013	0.050	0.004	0.248	0.178	0.199
Nonstriated muscles (averages).....	80.6	0.067	0.243	0.008	0.032	0.004	0.171	0.087	0.081	0.081	0.328	0.011	0.044	0.007	0.283	0.217	0.184
Striated muscles (Katz's averages).....	72.9	0.254	0.156	0.006	0.008	0.028	0.048	0.204	0.213	0.306	0.210	0.008	0.011	0.047	0.123	0.510	0.487

In explanation of these differences between the two classes of muscle the possibility of a considerable admixture of blood and lymph in the nonstriated tissue at once suggests itself. The low content of hæmoglobin excludes any considerable contamination with blood; and the iron content also fails to suggest any marked admixture. Urano¹ has calculated that one-sixth of the volume of frog's skeletal muscle consists of interstitial fluid. If it is recalled that the nonstriated muscles were shown above (p. 488) to be far richer in connective tissue (and therefore presumably also in lymph spaces) than skeletal muscle, one might be inclined to attribute the characteristic relation of the salts found above to the admixture of lymph with the normal salts of the muscle tissue proper. Urano assumes that the sodium ordinarily found in frog's skeletal muscle is present entirely in the interstitial muscle lymph, since it can be completely removed by suspending the tissue in isotonic sugar solution.

A comparison of the composition of fresh pig's muscle of different types with blood serum (resembling lymph) of the same species, indicates that the assumption of an admixture of lymph may explain the higher content of sodium and chlorine and the lower percentages of potassium, magnesium and phosphorus. It fails, however, to account for the noticeably higher content of calcium, both absolutely and relative to magnesium. For the percentage of calcium in the fresh muscle (ranging from 0.022 to 0.042 per cent) is considerable larger than that in either plasma (containing 0.009 per cent calcium) or skeletal muscle (0.008 per cent calcium).

TABLE III.

Comparative composition of pig's muscle and blood serum (parts per 100).

Source.	K ₂ O.	Na ₂ O.	Fe ₂ O ₃ .	CaO.	MgO.	Cl.	P ₂ O ₅ .	H ₂ O.
Nonstriated muscle (Saiki) . .	0.081	0.328	0.011	0.044	0.007	0.171	0.184	80.6
Skeletal muscle (Katz)	0.306	0.210	0.008	0.011	0.047	0.048	0.487	72.9
Blood serum (Abderhalden) .	0.027	0.425		0.012	0.004	0.363	0.020	91.8

The striking peculiarity of nonstriated muscle is its property of tonic contraction, as yet unexplained, and its tendency to auto-

¹ Urano: *Zeitschrift für Biologie* 1, p. 227, 1907.

matic rhythmic activity. Calcium ions distinctly facilitate these features. Thus Stiles¹ has shown that calcium tends to heighten the tonus of frog's œsophagus, and that precipitants of calcium cut short the rhythmic contractions. Other illustrations might be cited. At present, however, it will suffice to draw attention to a possible physiological significance of the relatively high Ca content of nonstriated muscle. Further speculation seems unwarranted.

Rigor and stroma-formation. The occurrence of true rigor in nonstriated muscle has been debated.² de Zilwa maintains that the *retractor penis* muscle of the dog does not undergo rigor mortis, and Saxl has made the statement more general for nonstriated muscle, basing his conclusion on experiments with the uterus musculature of the cow. Saxl has found, like previous investigators, that the rigor mortis of skeletal muscle is accompanied by a marked transformation of soluble, coagulable proteins into insoluble forms which are not redissolved when the tissue emerges from its stiffened condition. Both myosin and myogen (as termed in v. Fürth's classification) are involved in this coagulation. The experiments were made by comparing portions of muscle kept at ice-cold temperatures and examined at once with others which were allowed to stand some time during which the rigor progressively increased. The *heart* muscle showed only a slight decrease in soluble proteins (5 to 7 per cent) and a corresponding increase in the insoluble products. With nonstriated uterus muscle the content of these two fractions of the proteins was practically unchanged.

Saxl has found that the process of "stroma" formation (i. e., coagulation of soluble proteins) proceeds to the same extent in the cold as at room temperature in the course of twenty-four hours. Low temperatures merely delay the coagulation temporarily. We have compared the character of the proteins in stomach and bladder muscle kept twenty-four hours at room temperature and in snow with the data presented by Saxl, whose methods were

¹ Stiles: *American Journal of Physiology*, v, p. 338, 1901.

² Cf. Nasse: *Hermann's Handbuch der Physiologie*, i, p. 340, 1879; de Zilwa: *Journal of Physiology*, xxvii, p. 209, 1901; Vrooman: *Bio-chemical Journal*, ii, p. 363, 1907; Saxl: *Beiträge zur chemischen Physiologie*, ix, p. 17, 1907.

followed. The averages given below are taken from four concordant analyses on each type of muscle. Twenty or thirty grams were used in each estimation of the stroma and other proteins.

TABLE IV.

Comparative content of proteins in muscle (after twenty-four hours at different temperatures.)

Muscle used.	Temperature.	Total protein in the muscle.	Myo-in.	Myogen.	Total concu- lable protein.	Insoluble pro- tein (stroma).
		<i>per cent.</i>	<i>p. ct.</i>	<i>per cent.</i>	<i>per cent.</i>	<i>per cent.</i>
Pig's stomach.....	snow	13.8	1.1	31.4	32.5	67.5
	room	15.1	0.9	29.9	30.8	69.2
Pig's bladder.....	snow	12.0	2.6	23.0	25.6	74.4
	room	13.2	2.3	28.5	30.8	69.2
Rabbits' skeletal muscle (Saxl).....	ice		7.5	24.0	31.5	68.5
	room		6.5	22.0	28.5	71.5
Cow's heart muscle (Saxl).....	room		5.0	22.5	27.5	72.5

Transformation of glycogen. The paucity of glycogen in the nonstriated muscles examined suggested the question whether these are capable of transforming glycogen as is the case with skeletal and heart muscle. Kisch¹ has carried out an elaborate investigation of this property in striated muscles under a variety of conditions. No noteworthy differences were discovered in the various skeletal muscles. The heart musculature, however, is more efficient in this reaction. In the case of the bladder and stomach muscle the larger content of lymph (with its notable glycogen-digesting property) complicates the quantitative results when one attempts to refer them to the muscular elements themselves. Kisch's plan of investigation was followed in the trials reported here. The results are not strictly comparable with his, because the periods of digestion were somewhat dif-

¹ Kisch: *Beiträge zur chemischen Physiologie*, viii, p. 210, 1906.

ferent. The glycogen transforming capacity is shown to be present and not inconsiderable, especially at the low temperature (17°) maintained.

Transformation of glycogen by nonstriated muscle.

Muscle	Conditions of digestion trial.*	Glycogen solution added.	Glycogen found after 20 hours' digestion at 17° C.	Glycogen transformed.
		cc.	gm.	per cent.
Stomach, 50 gm.....	unboiled	50	0.229	59
"	boiled	50	0.555	
"	unboiled (control) }	none	trace	
Bladder, 50 gm.....	unboiled	50	0.224	60
"	boiled	50	0.562	
"	unboiled (control) }	none	trace	

*Toluene was present in every trial.

The figures presented indicated a transformation of glycogen equal to over 30 milligrams per 100 grams of muscle per hour; Kisch has recorded figures from 20 milligrams upwards for skeletal muscle under similar conditions.

This investigation was undertaken at the suggestion of Prof. Lafayette B. Mendel, to whom I am indebted for assistance and helpful advice.

ON THE OCCURRENCE OF A PHYTIN-SPLITTING ENZYME IN ANIMAL TISSUES.

BY E. V. MCCOLLUM AND E. B. HART.

(II. Contribution from the Agricultural Chemical Laboratory of the Wisconsin Experiment Station.)

(Received for publication, April 20, 1908).

The salts of anhydro-oxy-methylene-diphosphoric acid, or phytin, have been shown by several investigators¹ to be widely distributed in plants. Hart and Andrews² have shown that inorganic phosphorus does not occur in vegetable feeding-stuffs in appreciable quantities. Wheat bran, which contains $1\frac{1}{2}$ per cent of phosphorus, was found to be essentially free from inorganic phosphates, the whole of the phosphorus being present as salts of phytic acid. This discovery led to a number of investigations by biological chemists in order to find what rôle this new organic phosphorus body plays in metabolism.

Gilbert³ and his collaborators and later Mendel and Underhill⁴ have shown that the salts of phytic acid are not toxic when administered *per os* and that very large quantities are necessary when administered intravenously, intraperitoneally or subcutaneously to produce disturbing symptoms. The latter observers found the free acid much more toxic. Scofone⁵ observed that the phytin ingested is excreted largely as inorganic phosphoric acid, and this result has been confirmed by Giascosa⁶ and by

¹ Posternak: *Compt. rend de la soc. de biol.*, lv, p. 1190, 1903; Patten and Hart: *Amer. Chem. Journ.*, xxxi, p. 564, 1904; Schulze and Costoro; *Zeitschr. f. physiol. Chem.*, xli, p. 476, 1904.

² Hart and Andrews: *Amer. Chem. Journ.*, xxx, p. 470, 1903.

³ Gilbert and Posternak: *La médication phosphorée*, Paris, 1903; Gilbert and Lippmann: *La presse médicale*, Septembre, 1904; G. Sécheret: thèse de Paris, 1904.

⁴ Mendel and Underhill: *Amer. Journ. of Physiol.*, xvii, p. 75, 1906.

⁵ Scofone: Abstract in *Biochem. Centralblatt*, iii, p. 606, 1905.

⁶ Giascosa: Abstract in *Biochem. Centralblatt*, iv, p. 572, 1905.

Jordan, Hart and Patten,¹ who found no evidence of phytin in the urine of cows after the ingestion of very considerable amounts. Sécheret² stated that the enzymes of the digestive tract do not alter phytin and suggested that it is probably decomposed in the intestinal epithelium. According to Jordan, Hart and Patten³ pepsin and trypsin are without action on phytin. Further than this the fate of this substance in the body has not been investigated.

Recently Suzuki⁴ has reported the existence of a phytin-splitting enzyme in rice bran.

With a view to finding where phytin is decomposed in the body we have examined several tissues for the presence of a phytase. The method employed was to digest the sodium salt of phytic acid, which was prepared by extracting wheat bran according to the method of Patten and Hart,⁵ with an aqueous or glycerine extract of the tissue under examination at 38° to 40° C. Both toluol and chloroform were added to keep down bacterial growth. After one or two days the mixture was heated, after dilution when necessary, and the proteids removed by coagulation with the addition of the smallest possible quantity of acetic acid. The phosphoric acid was then determined in the filtrate by the method of Hart and Andrews⁶ by precipitating, in the presence of a small quantity of nitric acid, with neutral ammonium molybdate solution. In all cases blank determinations were made on the tissue extract alone and on a solution of sodium phytate which were digested in a similar manner.

EXPERIMENTAL.

Fresh calves' liver was ground as finely as possible and treated with toluol water. After twenty-four hours it was filtered and the slightly bloody solution used for the digestion. The phytic acid was dissolved in water and neutralized by titrating with

¹ Jordan, Hart and Patten: *Amer. Journ. of Physiol.*, xvi, p. 268, 1906.

² Sécheret: *Loc. cit.*

³ Jordan, Hart and Patten: *Loc. cit.*

⁴ Suzuki: *Bull. of the Coll. of Agric., Tokyo Imper. Univ.*, vii, p. 503.

1907.

⁵ Patten and Hart: *Loc. cit.*

⁶ Hart and Andrews: *Loc. cit.*

sodium hydroxide using phenolphthalein as indicator. This solution was added to the tissue extract and, after the addition of the antiseptic, it was incubated at 40° C. and examined as above described.

The following table gives the results obtained by digesting sodium phytate with water extract of liver:

TABLE I.

No. of Exp.	15 cc. of 1 per cent sodium phytate + 200 cc. liver ext.	200 cc. liver extract alone.	15 cc. of 1 per cent sodium phytate solution alone.	Difference.
	<i>gram P₂O₅.</i>	<i>gram P₂O₅.</i>	<i>gram P₂O₅.</i>	<i>gram P₂O₅.</i>
1.....	0.0980	0.0504	0.0070	+0.0406
2.....	0.1225	0.0644	0.0101	+0.0480
3.....	0.1365	0.0553	0.0123	+0.0197
4.....	0.0344	0.0336	0.0087	+0.0221

Table II gives the results obtained in two experiments in which sodium phytate solution was incubated with a glycerine extract of liver:

TABLE II.

No. of Exp.	15 cc. 1 per cent sodium phytate solution + 50 cc. glycerine ext. of liver.	50 cc. glycerine ext. of liver alone.	15 cc. 1 per cent sodium phytate alone.	Difference.
	<i>gram P₂O₅.</i>	<i>gram P₂O₅.</i>	<i>gram P₂O₅.</i>	<i>gram P₂O₅.</i>
1.....	0.0584	0.0367	0.0140	+0.0077
2.....	0.0672	0.0367	0.0140	+0.0165

Table III gives the results obtained in three sets of experiments in which calves' blood was incubated at 40° C. with a 1 per cent solution of sodium phytate.

Experiments showed that small amounts of phosphorus could be added to the tissue extracts and recovered by the method of Patten and Hart after removal of the proteids by heating in solution faintly acidified with acetic acid.

TABLE III.

No. of Exp.	15 cc. 1 per cent Na phytate sol. + 50 cc. blood diluted to 200 cc.	50 cc. blood alone diluted to 200 cc.	15 cc. 1 per cent sodium phytate solution diluted to 200 cc.	Difference.
	gram P_2O_5 .	gram P_2O_5 .	gram P_2O_5 .	gram P_2O_5 .
1.....	0.0423 0.0364	} 0.0150	0.0087	{ +0.0186 +0.0127
2.....	0.0364 0.0322	} 0.0119	0.0087	{ +0.0158 +0.0116
3.....	0.0315 0.0217	} 0.0098	0.0084	{ +0.0133 +0.0035

The above results indicate, we believe, that the liver and blood have the property of cleaving the salts of phytic acid with the production of inorganic phosphoric acid. The wide distribution of inosite in the tissues renders it impossible for us to say from experiments yet made whether this is also produced in this cleavage. These results are in accord with those of Mendel and Underhill,¹ who showed that the intestine is not necessarily involved in the excretion of the metabolic products of phytin in certain animals, and also with the conclusions of Scofone, that the enzymes of the digestive tract do not alter phytin. We have examined ptyalin, pepsin and trypsin and have confirmed Scofone's results.

Experiments made with extracts of muscle and kidney did not give results which pointed toward the presence of a phytase in these tissues.

¹ Mendel and Underhill: *Loc. cit.*

THE EFFECT OF DIET ON THE AMYLOLYTIC POWER OF SALIVA.

By C. HUGH NEILSON AND D. H. LEWIS.

(From the Physiological Department of St. Louis University.)

(Received for publication, April 20, 1908)

Neilson and Terry¹ found, in their work on the effect of diet on the saliva of dogs, that a carbohydrate diet produces a saliva in dogs with considerable amylolytic power; that on a meat diet this power was much lessened or entirely absent. This has been flatly contradicted by the experiments of Mendel and Underhill,² and Garry.³

The object of these experiments is to try the effect of diet on human saliva. The most of the work was done during the spring and summer of 1906. The results were reported at the meeting of the Physiological Section in New York in 1907. The work has been recently repeated. In March, 1907, L. G. Simon⁴ reported some experiments on human saliva, in which he mentions the action of diet on the amylolytic power of the saliva. He finds that the saliva of an individual on a carbohydrate diet is more powerfully amylolytic than the saliva of an individual on a mixed diet. He tested the saliva every hour and finds that on a carbohydrate diet the saliva maintains its high amylolytic power much longer than the saliva secreted during and after a mixed diet. On a protein diet the amylolytic power of the saliva is less powerfully amylolytic than the saliva of a mixed diet, but that it may reach a somewhat higher power one to three hours after the mid-day meal. These results are in keeping with my results, although in my experiments the saliva was tested on successive days.

¹ Neilson and Terry: *Amer. Journ. of Physiology*, xv, p. 406, 1906.

² Mendel and Underhill: *Ibid.*, iii, p. 135, 1907.

³ Garrey: *This Journal*, iii, p. xl, 1907.

⁴ L. G. Simon: *Journal de physiologie et de pathologie general*, ix, p. 261, 1907.

Method. A definite (10 cc.) quantity of saliva was always collected, the mouth being first rinsed with distilled water. The saliva for the afternoon experiments was always collected at 2:30 p.m. In the experiments carried out in the morning the saliva was collected at 10 a.m. As a control the saliva of the individuals to be experimented upon was tested for four days. During this time the subjects, generally students, were on a mixed diet and at the regular student duties. One set (three students) were placed on a carbohydrate diet and another set on a protein diet. The carbohydrate diet consisted of potatoes, bread, cereals of all kinds, and vegetables with a rich carbohydrate content. The protein diet consisted of all kinds of meats prepared in all ways, and eggs in any form. The quantity of food was not limited. The subjects kept up their ordinary duties. Coffee, tea, milk or beer were allowed, if the subjects were accustomed to drinking them. Regularity of meals was insisted upon.

The saliva was tested twenty-four hours after the diet was commenced and for each successive day at the same time as long as any given diet was kept up.

The saliva was collected and filtered through cheesecloth. Two and one-half cc. were then measured out accurately with a volumetric pipette. This was diluted up to 100 cc. with distilled water. Ten cc. of this dilute saliva was placed in an Erlenmeyer flask of 250 cc. capacity, containing 75 cc. of 2 per cent arrow-root starch paste made up to 90 cc. with distilled water. The contents of the flask had previously been warmed to 37.5° before the saliva was added. The contents were then thoroughly shaken and placed in an incubator registering $37\frac{1}{2}^{\circ}$ C. and kept there for twenty minutes. At the end of this time the flasks were taken out and the contents boiled and made up to 100 cc. The maltose produced was at once determined by the Haines titration method. Before the titration the flasks were cooled to room temperature. In the titrations a rough determination was first made and the contents diluted so that each contained about the same percentage of maltose. The amount of maltose was calculated and the per cent of increase or decrease calculated on the control as a basis.

Experiment.	Control amount of maltose. Average for 3 days.	Diet.	PERCENTAGE CHANGE CALCULATED ON AMOUNT OF MALTOSE IN CONTROL.			Diet.	Percentage change calculated on last day of preceding diet.	Diet.	Percentage change calculated on last day of preceding diet.
			1st day.	2d day.	3d day.				
W.....	120 mg. }	Protein	-13	-13	-10	Changed to carbohydrate	+7.8	Changed to protein	-22
L.....	120 mg. }		-7½	-8	-10		+49		-39
K.....	116 mg. }	Carbohydrate	+8.6	+30	+62	Changed to protein	-52	Changed to carbohydrate	+42
M.....	91 mg. }		+18	+20	+34		-25		+65

From the tabulated results, it is seen that on a protein diet the percentage of sugar is decreased as shown by the percentage column; for instance, in the experiment marked W there is a decrease of 16 per cent in the amount of sugar produced on the third day of the protein diet calculated on the amount produced in the control as a basis; also that two days on a protein diet this same subject shows an increase of 78 per cent in the amount of maltose produced over that on the last day of the preceding protein diet; that after a change to a protein diet, there is a decrease of 39 per cent over that of the last day of the preceding carbohydrate diet. These changes from day to day are seen to be gradual. The most marked and sudden change is found when the carbohydrate diet is used. The change produced by the protein diet is more gradual. The other experiments show the same thing, with a difference only in the percentages.

The results given in the table are only average results. In many of the other experiments, the change was as great as 150 per cent. Diets were given in all, to 25 different individuals and they all show the same general result as on those given in the table.

In Fig. 1, the change is shown by a curve representing the percentage change for every twenty-four hours. Also the change in the direction of the curve on different diets is seen. The distance between the heavy lines reading from left to right represent twenty-four hour periods at the end of which the quantitative determinations were made.

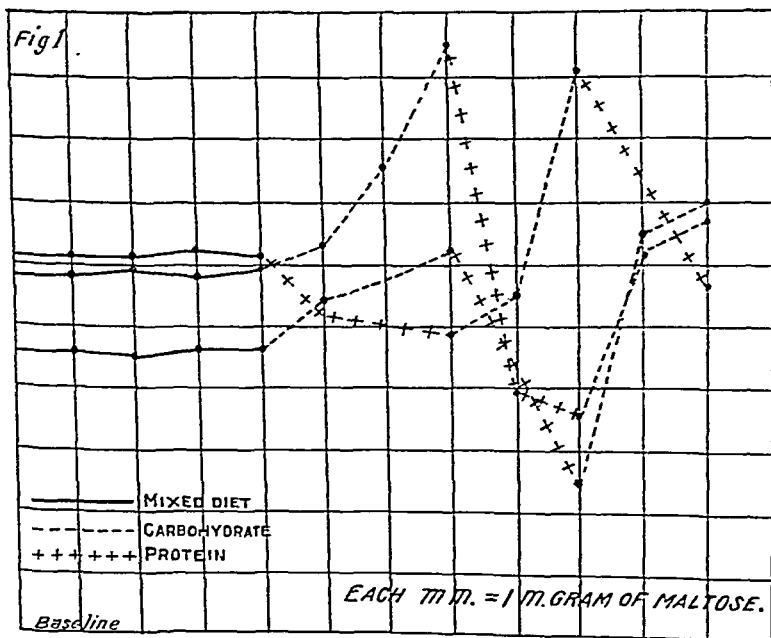
In another set of experiments the attempt was made to prolong the diet. In one set, three individuals were kept on an excess of carbohydrate, for six days. The amount of maltose produced on the fifth and sixth day of this diet was double that produced by the control or normal saliva.

After the change to the protein diet the sugar gradually decreased until the eighth day. This decrease was 64 per cent less than that of the last day of carbohydrate diet. The ninth, tenth and eleventh days showed practically the same as that of the eighth. The explanation of this latter fact is not clear. However, the meat or protein diet grew irksome to the individual and the appetite was not good. This may be the explanation. The results in the other two subjects were similar but not so

marked. The effect of the diet varies a great deal in different individuals.

The best and most striking results were obtained in those individuals to whom the diet did not become irksome. The response to any given diet was most pronounced when the subject had as much variety in the diet as possible.

This change in the amylolytic power of the saliva produced by different diets is difficult to explain. The food is the natural



stimulus to the flow of the saliva. It is within the bounds of reason to say that the stimulus of one kind of food in excess might stimulate the nerve endings in the buccal mucosa, and thus the salivary glands may be stimulated reflexly to the secretion of a greater amount of ptyalin, or even a ptyalin with a greater activity, when an excess of carbohydrates is given, and the reverse on an excess of protein food.

The saliva may be of greater concentration in the case of the carbohydrate diet than a protein diet and this would result in a

relative increase in the amount of the ptyalin in a given quantity of saliva.

Dryness of food will augment power of saliva according to Scheunert and Illing.¹ If the food causes a stimulation of the salivary glands, and this changes the chemical constituents of the saliva, it would seem that the change in amylolytic power should appear early in the course of the diet. No attempt was made by us to determine this point, as the saliva in no case was tested earlier than twenty-four hours after the beginning of the diet. It is known that there is an increase in the sugar content of the blood on a rich carbohydrate diet.

It is then conceivable that the increase might stimulate the salivary glands to a greater activity. Some work is now being done to determine this point. Whether this change in the amylolytic power of the saliva due to diet should really be called an adoption to diet is immaterial. At least there is a change either in the amount of ptyalin or in its activity, or in the concentration of the saliva, which enables more or less starch to be digested with a given quantity of saliva according to the diet.

What better name can be given than adaptation?

Carlson and Ryan² have found that in normal saliva there is always a small amount of glucose present. This certainly can not be the explanation of the facts by our experiments. Our method of control rules out this explanation.

¹ Scheunert and Illing: *Centralblatt f. Physiol.* No. 10, 1906.

² Carlson and Ryan: *Amer. Journ. of Physiol.*, xxi, p. 30, 1908.

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